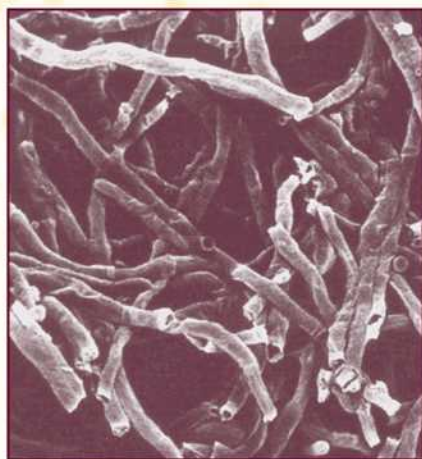


Fungal Biotechnology in Agricultural, Food, and Environmental Applications



edited by
Dilip K. Arora

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Fungal Biotechnology in Agricultural, Food, and Environmental Applications

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Preface

The study of fungal biotechnology is proceeding at an unprecedented rate with an array of new tools to generate a wealth of disciplines and subdisciplines. By means of modern biotechnology, fungi have justified their practical application to varied domains of human enterprise, and thus promise considerable potential in the agricultural, food, and environmental spheres. The successful application of fungal biotechnological processes in these areas requires the integration of a number of scientific disciplines and technologies. These may include subjects as diverse as agronomy, chemistry, genetic manipulation, and process engineering. The practical use of newer techniques such as genetic recombination, bioinformatics, and robotics has revolutionized modern biotechnology-based agri-food industries, and created the enormous range of possible applications of fungi.

Tremendous biodiversity of agriculturally important fungi exists—the benefit of which is not fully harnessed. The level of technology required to take full advantage may range from the simple introduction of a single fungus in biocontrol processes to the extensive manipulation of the organism that facilitates overproduction of a particular enzyme or metabolite. In modern agri-industry, fungi offer many established beneficial roles, particularly as biofertilizers, mycorrhizae, and biocontrol agents of pathogens, pests, and weeds. As pathogens, fungi represent a heavy negative impact on human health, agriculture, and environment. In agriculture, annual crop losses by phytopathogenic fungi in the field and also during post-harvest exceed 200 billion Euros, and in the United States alone, over \$600 million are spent annually on agricultural fungicides. The balance of beneficial and detrimental effects is reflected in many other areas of agriculture and horticulture. Fungi that inhabit tropical or temperate soils, as mycorrhiza, endophytes, phytopathogens, entomopathogens, or simple saprophytes, are significant resources in transformation of biological matter, and they offer many bioproducts including secondary metabolites, antibiotics, and catabolic enzymes of enormous potential.

The world has won a crucial battle in the area of food security, but the war is still on. A total of 800 million people—that is, one of every six persons in the developing world—do not have access to food. One-third of all pre-school-age children in the developing countries face food insecurity. In the food and feed arena, fungi are historically important as mushrooms and fermented foods and in baking and brewing. Such roles are supplemented by the provision of fungi to offer food processing enzymes and additives, and more recently the development of protein-based foodstuffs from filamentous fungi. On the detrimental side, fungi cause extensive spoilage of stored and processed foodstuff. Through direct pathogenesis and biodeterioration of foods and other agricultural commodities, fungi cause considerable economic consequences as well. In these cases, techniques developed from biochemistry and molecular biology can be deployed to analyze the relevant processes, and to evolve tools for the detection, characterization, and tracking of the organisms involved. Although such endeavors may seem rather far removed from the traditional definition of fungal biotechnology, the information derived can be pivotal in understanding the underlying intricate processes, and arriving at suitable control measures.

The utilization of fungi in the environment is a more recent development, and can have particular association with both food and agriculture, with fungal remediation of land having implications for biofertilizers, mycorrhizae, and food crop

development, among many other considerations. The degradative activities of fungi have also been harnessed in programs related to bioremediation of contaminated land, treatment of industrial wastes, and biotransformation of specific compounds. Many of the applications of fungal biotechnology in these areas rely not on identifying new activities but in harnessing and expanding roles that the fungi undertake normally in the environment.

Several books on the role of fungi in agricultural, food, and environmental applications have appeared since the 1990s. However, subjects relating to these areas are so broad that no single book can provide all the available information. Consequently, this book complements the others by providing valuable information that is not available elsewhere. The book encompasses a broad range of information on biotechnological potential of entomopathogenic fungi, ergot alkaloids, fungi in disease control, the development of mycoherbicides, control of nematodes, control of plant disease, strategies for controlling vegetable and fruit crops, mycotoxigenic fungi, development of biofungicides, production of edible fungi, fermented foods, and high-value products such as mycoprotein, yeasts in the wine industry, the role of fungi in the dairy industry, molecular detection of fungi in food and feeds, antifungal food additives, the importance of fungi in forest and arid ecosystems, the role of fungi in the biomineralization of heavy metals, bioconversion of distillery waste, decoloration of industrial waste, and fungal degradation of cellulose, hydrocarbons, dye water, and explosives.

Together with its companion publication, the *Handbook of Fungal Biotechnology, Second Edition* (Marcel Dekker, 2004), this incomparable book reigns as the top source on the role of fungi in agriculture, food technology, and environmental applications. The book will be useful for teachers and students, in both undergraduate and graduate studies, in departments of agricultural microbiology, food science, food technology, food engineering, microbiology, environmental sciences, botany, bioengineering, plant pathology, mycology, and, of course, biotechnology. In addition, the book will be useful for agri-food producers, research establishments, and government and academic units.

No work of this magnitude can be accomplished without the support and contributions of many individuals. I am deeply indebted to my colleagues and associate editors who have assisted me throughout the production of this book. I appreciate the hard work of authors for their up-to-date discussions on various topics and immense persistent cooperation. My gratitude is expressed to my teacher J. L. Lockwood. I thank Ms. Sandra Beberman (Vice President) and Ms. Dana Bigelow (Production Editor) at Marcel Dekker, Inc., for their dedicated assistance and advice in editorial structuring at all stages of the production of this book.

Dilip K. Arora

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Biotechnological Approaches in Plant Protection: Achievements, New Initiatives, and Prospects

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1 CHALLENGES FOR FOOD SECURITY IN THIS CENTURY AND BEYOND

Today we face many critical issues in agriculture: (a) an exponentially growing human population; (b) recurrent famine; (c) the destruction of natural landscapes such as tropical rain forests to extend agriculture to previously unused lands; (d) the exodus of human civilization from rural communities to cities; (e) the destruction of environmental quality resulting from exposure to agrochemicals, erosion of soils and salinization of soils as well as exhaustion and contamination of fresh water resources; (f) the loss of biodiversity through monocropping and the destruction of natural habitats; (g) the reliance of agricultural production, transport, and storage systems on fossil fuel; (h) the acquisition and concentration of agricultural wealth by multinational corporations; and (i) an issuant lack of knowledge by a growing proportion of human civilization on how to cultivate, prepare, and preserve food. The United Nations Food and Agriculture Organization predicts that agricultural productivity in the world will be able to sustain the growing human population by 2030 but hundreds of millions of people in developing countries will remain hungry and environmental problems caused by agriculture will remain serious (FAO 2002). By 2025, 83% of the expected global population of 8.5.2 billion will be in the developing world (United Nations 2002). The social consequences are obvious. Food is a basic human need and right. How can we sustain the food needs of the earth's biotic community in the 21st century and beyond while preserving environmental quality and the diversity and quality of life on earth (Time, August 26, 2002)? What solutions can biotechnology provide to address these problems (Khush and Bar 2001)?

In the last century, the Green Revolution addressed the food needs of the human population through the development of high yielding and early maturing varieties that performed under favorable conditions of nutrition and moisture (Khush 2001). Prior to this time, increased production was dependent on expansion of land area for crop production. In recent years, yield gain through breeding has not kept pace with population growth (Serageldin 1999). Furthermore, genotype is not the only factor limiting productivity. Abiotic and biotic stress factors also contribute to losses in yield both pre and postharvest. For example, in Asia these technical constraints on rice production may reduce production by 23% (Evanson et al. 1996). Socioeconomic constraints also contribute to practices that affect yield. Ninety percent of the world's rice is grown in Asia on small farms with limited resources. Thus, decisions are made based on economics rather than achieving technically optimum yields. Despite much research on the application of biotechnology to solve these technical production constraints, biotechnology has had limited impact to date on rice production in Asia (Houssain 1997). This has been due in part to the reluctance to adopt the use of genetically modified organisms (GMOs) in most countries of Asia. China is the only Asian country that has embraced biotechnology on any notable scale as a solution to technical production constraints in agriculture (Huang et al. 2002; Pray et al. 2002). For example, over four million smallholders have been able to increase yield and reduce pesticide costs and adverse health effects of applying pesticides by using transgenic Bt-cotton (Pray et al. 2002). Another constraint relates to the complexities of ownership rights that can delay and discourage the scientific application of biotechnology discoveries and their transfer to the market place (Kowalski et al. 2002). Finally, public concern about the safety of consuming GMOs has left tons of food aid containing transgenic corn untouched in

Zimbabwe and Zambia despite the prediction that 13 million people are now at risk of famine due to severe drought in Southern Africa (Paarlberg 2002). Organic growers worldwide have rejected GMOs as an allied technology to disease and pest management. Late blight in potato is still very difficult to control in these farming systems (Mader et al. 2002).

Biotechnology as applied to plant protection against fungal pathogens has seen three phases of development over the last 20 years: (a) the application of molecular markers to marker-assisted breeding and the map-based cloning of genes associated with disease resistance and the plant defense response as well as to study fungal pathogenesis and host recognition; (b) the development of routine methods for stable and transient transformation of plants and fungi with foreign genes; and (c) the application of New Biology approaches to study plant growth and development and mechanisms of plant response to abiotic and biotic stresses and fungal pathogenesis. The purpose of this short review is to provide a critical analysis of these recent biotechnological approaches to plant protection with emphasis on fungal pathogens.

2 TESTED STRATEGIES

2.1 Marker-Assisted Breeding and Map-Based Cloning of Genes

2.1.1 Molecular Maps

With the advent of recombinant DNA technology came the application of cloned DNAs as probes to genomic DNA of the source organism and the revelation that different alleles could be detected between individuals based on restriction fragment length polymorphisms (Helentjaris et al. 1985) (Figure 1). Genetic maps based on these and other types of molecular markers (Table 1) were developed for many organisms including crop plants such as rice (McCouch et al. 1988; <http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html>), lettuce (Kesseli et al. 1994), tomato (Tanksley et al. 1992), alfalfa (Brouwer and Osborn 1999), and Brassica spp. (Kole et al. 2002), among others. Likewise molecular

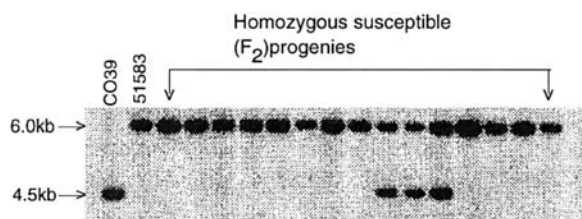


Figure 1 Cosegregation of a RFLP marker (R-23 16) with *Pi-CO39(t)* locus in homozygous F₂ susceptible progenies. Genomic DNA of CO39 (R, resistant), 51583 (S, susceptible) and F₂ progenies was digested with *Dra*I, blotted and probed with R-2316. Recombinant progenies show DNA fragments from both parents. Phosphoimage of Southern blot is shown.

maps were developed for key fungal pathogens such as *Magnaporthe grisea* (Farman and Leong 1995; Nitta et al. 1997; Skinner et al. 1993; Sweigard et al. 1993), *Phytophthora infestans* (van der Lee 2001), and *Leptosphaeria maculans* (Pongam et al. 1988). These studies also began to reveal the complexities of these genomes in terms of repeated DNAs, their function as transposable elements (Goff et al. 2002; Hamer et al. 1989; Kachroo et al. 1997), their distribution within the genome (Goff et al. 2002; McCouch et al. 1988; Nitta et al. 1997; Yu et al. 2002), and their role in genome evolution and host recognition (Farman 2002; Farman et al. 2002; Kang et al. 2001; Song et al. 1997; 1998). Comparative maps were generated in plants by mapping markers across genera and showed considerable synteny within families of plants (Ahn and Tanksley 1993; Bennetzen and Freeling 1993; Chen et al. 1997; Dunford et al. 1995; Gale and Devos 1998; Hulbert et al. 1990; Saghai Maroof et al. 1996; Tanksley et al. 1992). The mapping of phenotypic markers, both native and induced by mutation, followed closely behind and yielded precise information on the chromosomal location of genes important to plant defense (Ronald et al. 1992; Wang et al. 1995) and fungal host specificity (Dioh et al. 2000; Smith and Leong 1994; Sweigard et al. 1993) and led to their cloning by chromosome walking (Cao et al. 1997; Farman and Leong 1998; Martin et al. 1993; Orbach et al. 2000; Song et al. 1995; Sweigard et al. 1995). The cloning of a plethora of disease resistance genes from many plant species has shown that they belong to a small number of structural classes (Brueggeman et al. 2002; Chauhan and Leong 2002; Dangl and Jones 2001; Meyers et al. 1999; Xiao et al. 2001) (Figure 2). By contrast, the predicted structures of fungal cultivar specificity genes are quite diverse (Bohnert et al. 2001; De Wit and Joosten 1999; Orbach et al. 2000; Sweigard et al. 1995).

These studies have been complemented by the mapping of candidate genes such as the PR (pathogenesis-related) proteins in plants that were discovered from differential expression of RNA and protein during plant infection (Muthukrishnan et al. 2001) or resistance gene analogs based on the conserved structural features of disease resistance genes (Boyko et al. 2002; Chauhan et al. 2002; Faris et al.

Table 1 Molecular markers used in mapping of traits

Marker	
RFLP	Restriction fragment length polymorphism
RAPD	Random amplified polymorphic DNA
APD	Amplified polymorphic DNA
CAP	Cleaved amplified polymorphism
AFLP	Amplified fragment length polymorphism
Microsatellite	Polymorphism based on different numbers on mono, di, tri, or tetranucleotide repeats
CDNA-AFLP	cDNA amplified restriction fragment length polymorphism

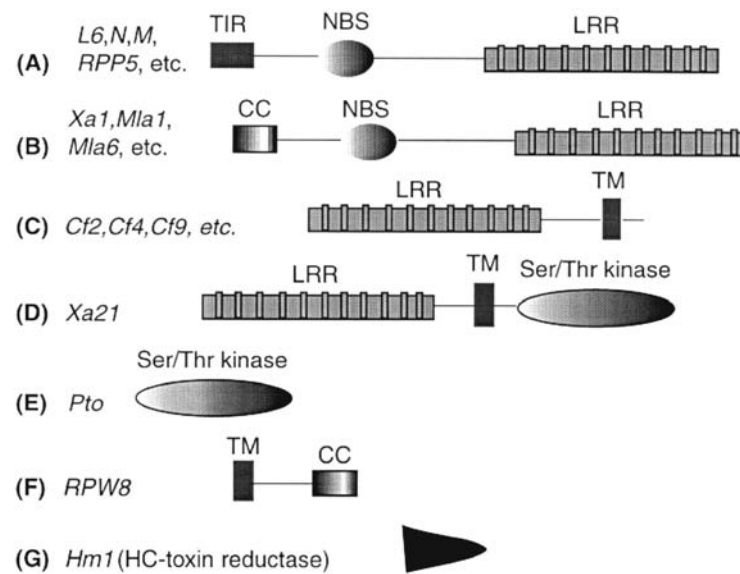


Figure 2 Different classes (A–G) of plant disease resistance genes [reviewed in Chauhan and Leong (2002)]: Genes in classes A and B are cytoplasmic proteins differing only in their N-terminal domains; Class C genes encode putative transmembrane molecules with an extracellular LRR domain; *Xi21* is a transmembrane protein with an extracellular LRR domain; *Pto* is a cytoplasmic Ser/Thr kinase; *RPW8* contains a putative N-terminal TM domain and a CC domain; *Hm1* is a unique enzyme that detoxifies a fungal toxin; Abbreviations for domains: TIR, Drosophila Toll/Human Interleukin-1 receptor; CC, Coiled-coil; NBS, Nucleotide binding site; LRR, Leucine-rich repeat; TM, Transmembrane; Ser/Thr, Serine/threonine kinase.

1999; Gebhardt and Valkonen 2001; Huang and Gill 2001; Li et al. 1999; Shen et al. 1998; Speelman et al. 1998). This analysis has been particularly well advanced in wheat and its relative *Aegilops tauschii*. Resistance and defense response genes in *A. tauschii* are localized in clusters primarily in distal/telomeric regions of the genome (Boyko et al. 2002) while in Chinese spring wheat defense response genes are localized in clusters and/or at distal regions of chromosomes (Li et al. 1999). In many cases, these genes or gene homologs have been correlated with loci that affect quantitative or single gene resistance in the respective plants. For example, QTLs with large effects in wheat were shown to contain RGAs or clusters of defense response genes such as catalase, chitinase, thaumatins, and an ion channel regulator (Faris et al. 1999). Similar results are emerging in the genomes of potato (Gebhardt and Valkonen 2001), Arabidopsis (Speelman et al. 1998) and pepper (Pflieger et al. 2001).

Efforts are underway to functionally characterize 179 NBS-LRR-encoding genes that may encode disease resistance genes in the Arabidopsis genome (Figure 2). These have been organized into subclasses and their distribution mapped to the chromosomal sequence (Michelmore 2002; www.nibh-rs.ucdavis.edu). A publicly available, draft ordered sequence of the rice genome is anticipated by the end of 2002 (<http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/seqcollab.pl>) and will allow comparisons to be done across syntenic regions of grass genomes (<http://www.gramene.org/>). The unordered draft sequence of rice varieties Nipponbare

and 93-11 has revealed numerous NBS-LRR-containing sequences as well as sequences potentially encoding other minor classes of R genes and Arabidopsis genes known to control defense response signal transduction (Goff et al. 2002; Yu 2002). Preliminary studies based on conservation of RGAs in comparative maps of the grasses have shown evidence for some conservation but also redistribution of this class of genes among the grasses (Leister et al. 1998). Likewise a detailed comparison of a syntenic region between barley and rice did not reveal any candidate resistance genes in rice that could be the ortholog of *Rpg1* in barley (Han et al. 1999; Kilian et al. 1997).

2.1.2 Differential cDNA-AFLP Screens

Differential cDNA-AFLP screening has been done to isolate hypersensitive response (HR)-specific genes to the *Cladosporium fulvum* elicitor Avr4 in tomato and has led to the isolation of a previously known and corresponding disease resistance gene cluster *Cf-4* as well as numerous new candidate genes involved in the HR response (De Wit et al. 2002; Takken et al. 2001). This method is a robust and inexpensive way to identify differentially expressed genes involving the digestion of cDNAs with two different restriction enzymes and the amplification of the resulting products after ligation to adapters for these enzymes. The sizes of the resulting amplicons are measured by gel

electrophoresis and resulting fragments can be excised and sequenced. Comparison of this method with differential display has shown the cDNA-AFLP method to be superior (Jones and Harrower 1998). Using the cDNA-AFLP technique, Durrant et al. (2000) found a strong coincidence between the expression of genes involved in race-specific resistance and the wound response in tobacco cell cultures. Collectively, these candidate genes will provide additional markers for studies of disease resistance traits in the potato and tomato genomes. Infection-specific cDNA-AFLPs have also been identified in *Arabidopsis thaliana* inoculated with *Peronospora parasitica* (van der Biezen et al. 2000). Interestingly, most fragments were derived from the fungal pathogen showing the power of this method to study genes expressed in the pathogen, which in many cases may represent a minor component of the mass of the tissue studied.

Previous genetic studies by Valent et al. (1991) have shown that infection of some grass hosts by *M. grisea* is a quantitatively inherited trait. The cDNA-AFLP approach would allow for the identification a unique set of cDNA-AFLPs in each progeny showing varying degrees of pathogenicity and in some cases segregating with pathogenicity. This approach has been recently used to create genome-wide transcription maps of *Arabidopsis* and potato and study inheritance of the cDNA-AFLPs in segregating populations (Brugmans et al. 2002). Thus phenotypes can be directly associated with molecular genotypes and candidate gene fragments can be excised from gels for further analysis. We are using this approach in an attempt to identify major and minor genes controlling resistance to blast and drought tolerance in *Eleusine coracana*.

Computational methods for relating the size of the AFLP restriction fragment products to the predicted restriction fragment products from sequenced cDNA libraries have been developed and used to identify putative, infection stage-specific, pathogenicity factors from the plant pathogenic nematode *Globoderu rostochiensis* without need for sequencing the gel fragments (Qin et al. 2001). For those organisms having fully sequenced, annotated full length cDNA libraries such as *Arabidopsis* (Seki et al. 2002), this approach provides for the rapid functional classification of the cDNA-AFLPs.

2.1.5 Identification of QTL-Associated Genes

Very few QTL studies in plants have led to the cloning of a single gene within a QTL that is responsible for the variation seen [reviewed in Buckler and Thornsberry (2002)]. These few examples represent QTLs that had major effects on variation. Buckler and Thornsberry (2002) have proposed that association approaches should be also considered to provide improved resolution and to reduce the time of analysis as mapping populations are not needed since natural variation in a population is investigated instead. The resolution of association that can be obtained depends on the linkage disequilibrium (LD) structure of the population of organism being studied and some insight on candidate gene(s) to target. Studies on LD structure have shown that inbreeding plants

such as *Arabidopsis* have large LD structures on the order of 250 kb or 1 cM (Nordborg et al. 2002) while outbreeding plants like maize have very small LD structures in the order of kbs (Buckler and Thornsberry 2002). Using this approach, Thornsberry et al. (2001) were able to associate polymorphisms found in the *Dwarf8* gene of maize with variation seen in flowering time.

In *Saccharomyces cerevisiae* whose entire genome sequence is known, a QTL for high temperature growth (Htg) commonly found in clinical isolates was rigorously analyzed to identify the responsible genes (Steinmetz et al. 2002). Using reciprocal hemizygosity tests, involving selective gene disruption of candidate genes in both parental genomes and then forming diploid hybrids among these strains, three genes were found to contribute to the phenotype in the QTL interval. However, the alleles of two genes came from one parent strain while that of the third came from the other parent. By contrast attempts to employ natural sequence variation or mRNA expression levels determined from several natural isolates of yeast did not provide a clue to which gene(s) in the interval contributed to the phenotype. Thus employing LD and association by decent to accelerate gene identification in an interval may not always succeed and genetic studies will be required to study inheritance and create reciprocal hemizygotes in targeted regions of the genome. The use of allele-specific gene silencing methods (see below) may allow this to be done in the F1 generation of plants while targeted gene disruption methods can be used in fungi such as *Ustilago maydis* that have a stable diploid phase and facile gene knockout system. Transformation of haploid fungi with wild type and disrupted, endogenous or alternative alleles of candidate genes might be a useful strategy for those fungi that cannot form stable diploids. In fact these strategies have been used to unravel the complex functions of the east and west alleles of *b* mating type locus of *U. maydis* (Gillissen et al. 1992; Kamper et al. 1995). Gene silencing has been used in fungi such as *P. infestans* in which silencing of the fungal elicitor INF1 increased virulence on *Nicotiana benthamiana* (Kamoun et al. 1998).

2.1.5 Candidate Gene Validation

It should be emphasized that candidate genes are simply "candidate" genes and that confirmation of a gene's function with a genetically and/or expression-defined phenotype must be done by transformation and complementation tests (Farman and Leong 1998; Orbach et al. 2000; Song et al. 1995; Wang et al. 1999; Yoshimura et al. 1998). Recently gene silencing has also been successfully applied to study gene function in several plants (Azevedo et al. 2002; Baulcomb et al. 2002; Peart et al. 2002; Wesley et al. 2001). This method involves the cloning of a small fragment (~200 nucleotides) of a gene into an expression vector and transforming the plant [reviewed in Baulcomb et al. (2002)]. The resulting small RNA is made double stranded and then is digested into small dsRNA fragments (siRNA, small interfering RNA), which are thought to guide RNase to the

nascent wild-type gene transcript and causes it to be degraded thus leading to a net loss in the gene's expression. Direct bombardment of plant cells with dsRNA is also possible (Schweizer et al. 2000). In the examples described earlier, the specific genes *RARI* in barley, and Rx, N, Pto, and *EDS1* in *N. benthamiana* implicated in disease resistance signaling were silenced and found to be essential for the signaling process. This approach is being extended to the high throughput analysis of the HR candidate genes from tomato noted earlier (De Wit et al. 2002) as well as in a normalized cDNA library from *N. benthamiana* (Baulcomb et al. 2002). Interestingly, many genes in *N. benthamiana* were found in the preliminary round of analysis to affect the HR while not affecting pathogen growth while some genes were affected in both phenotypes when silenced. Moreover almost 1% of the genome appears to affect the HR response.

2.1.5 Integration of Molecular Biology with Classical Breeding

The application of molecular markers to traditional breeding has provided a powerful method to accelerate breeding as phenotypic tests are not essential and rapid DNA isolation methods using hole punch-sized pieces of leaf tissue are possible in young seedlings (Huang et al. 1997). Thus tightly cosegregating or gene(allele)-specific markers can be followed and confirmation of phenotype can be done on a selected set of plants within a population that are destined for further crossing. Phenotypic validation is essential as recombination, gene conversion or other confounding events can take place, even within the gene being studied, leading to an inaccurate scoring based on markers alone. As we learn more about the function of plant genes and specific alleles of these genes in disease resistance through mapping and functional tests, we can anticipate the increased application of molecular markers and gene chips (see later) to the breeding of disease resistance in plants. In particular, it will be interesting to know what contribution pathogenesis-related proteins, which are generically present in all plant genomes, make to quantitative resistance. Is expression more efficient in some genomes than others because of the gene's placement in clusters and/or their specific transcription regulatory elements and/or their duplication or absence in some genomes and/or the efficacy of specific alleles? Likewise, what is the genetic and molecular basis of host specialization in the fungi? The location of many disease resistance and defense genes at the ends of chromosomes in wheat may affect their stability through recombination and chromosome breakage as well as their expression through unique chromatin organization (Faris et al. 2000). The telomeric location of *AVR1-PITA* in *M. grisea* has been shown to contribute to its instability leading to strains with increased virulence (Orbach et al. 2000). The *BUF1* gene of *M. grisea* appears to be readily deleted in one parental chromosome by intrachromosomal recombination of repeated DNA flanking the locus as a result of mispairing of homologous chromosomes during meiosis (Farman 2002). In *Alternaria alternata* a conditionally

dispensable chromosome controls production of a host-specific toxin (Hatta et al. 2002). Likewise Han et al. (2001) found that genes required for pathogenicity on pea are present on a dispensable chromosome of *Nectria haematococca*.

Exploitation of the multitude of novel genotypes found in plant germplasm now available in gene banks will often require the methods of molecular mapping and candidate gene isolation described above to identify the genes that contribute to these unique phenotypes (Fulton et al. 1997; Tanksley and McCouch 1997; Xiao et al. 1998). This is true even for plant genomes for which the entire genome sequence is known, thus allowing candidate genes to be isolated in related genomes. For example, the short stature mutation *sg1* found in green revolution rice variety IR8 encodes a mutant biosynthetic gene for gibberellin while the semidwarf phenotype in green revolution varieties of wheat is conferred by mutations in the gibberellin signaling pathway (Sasaki et al. 2002). In addition, many previously unidentified genes have been found in every genome that has been sequenced (Goff et al. 2002; Yu et al. 2002). In the case of disease resistance genes, the functions of only a few are known in each sequenced genome and not all LRR-containing coding sequences are likely to function in disease resistance. For example, a LRR receptorlike transmembrane protein kinase gene, that is gibberellin-induced and specifically expressed in growing tissues of deep-water rice, may function in hormone signaling (van der Knaap 1999). The genetic location of disease resistance genes must be determined in the genome that contains the functional gene if the reference genome sequence lacks a functional copy. These tenets also apply to the identification of fungal genes involved in plant recognition. Examples exist for the complete absence of a recessive gene in fungal strains that have lost cultivar specificity (van den Ackerveken et al. 1992; Farman et al. 2002). Furthermore, the recently released genomic DNA sequence of *M. grisea* having 7X coverage (<http://www-genome.wi.mit.edu/annotation/fungi/magnaporthe/>) does not contain the *AVR1-CO39* cultivar specificity gene (RS Chauhan, D Lazaro, and SA Leong, unpublished data). This genome sequence is thus useless without precise genetic mapping data for this *AVR* gene that can be used to identify in the reference, sequenced genome, a contiguous sequence spanning the genome between these markers. This sequence can then be used to develop new genetic markers and to probe libraries of a strain that does carry *AVR1-CO39*. In fact this *AVR* gene was originally cloned using a more laborious chromosome walking strategy in the genome of a strain carrying the functional gene (Farman and Leong 1998).

2.2 Transgenic Plants As a Tool for Plant Protection

Following clues from the molecular biology of plant-microbe interactions, many genes involved in disease resistance and

the defense response have been cloned from a variety of plants (Dangl and Jones 2001). A number of these genes have been tested for their ability to control fungal pathogens in transgenic plants grown in the laboratory and to a more limited extent in the field. In addition, natural or synthetic antimicrobial peptides and genes for resistance to pathogen-derived toxins have been introduced into plants. This work is thoroughly reviewed by Bent and Yu (1999); Rommens and Kishore (2000), and Melchers and Stuiver (2000). Only more recent studies providing significant new findings or extensions of this work will be considered here.

Current efforts have continued to focus on introduction of disease resistance genes, natural and synthetic antimicrobial peptides as well as selected enzymes such as chitinase and β -glucanase into several crop plants and the laboratory and field evaluation of these plants for disease resistance. Little has been reported in the literature on the field performance of these transgenic plants, although about 5% of all permits for field testing of transgenic plants in the United States over the last decade have been for transgenic plants having fungal resistance (Information Systems for Biotechnology 2002). Moreover, most permits have been issued to companies and this proprietary work is not yet in the public domain. No transgenic plants having fungal resistance have been approved for use as food and/or feed (AgBios 2002). Despite this seemingly limited progress, some promising

achievements have been made in the last few years and many major resistance genes associated with fungal disease resistance have been cloned or at least tagged with molecular markers (Brueggeman et al. 2002; Chauhan and Leong 2002; Gebhardt and Valkonen 2001). Thus, we can expect many new developments with regard to this field in the next decade. Furthermore, despite public concern about transgenic crops, the global adoption of transgenic crops continues to increase, particularly in the United States, where 74.8 million acres were planted in transgenic crops including corn, soybean, cotton, and canola in 2000 (Transgenic Crops 2002). This represented about 50% of the total soybean and cotton acreage planted in that year. The International Service for the Acquisition of Agri-Biotech Applications predicts that the world market for genetically engineered plants will be \$8 billion in 2005 and \$25 billion by 2010 (<http://nature.biotech.com>) (Figure 3). Plant pathogens cause \$30–50 billion dollars of loss annually in crop productivity (Baker et al. 1997) thus justifying this investment in biotechnological approaches to crop protection. The reduction in use of agrochemicals for disease control is another important incentive for this technology. Japanese growers spend more than \$600 million a year to control diseases on rice (Bonman 1998). Already, the reduction in insecticide use in China through use of Bt transgenic crops has impacted farmer income and health (Huang et al. 2002; Pray et al. 2002).

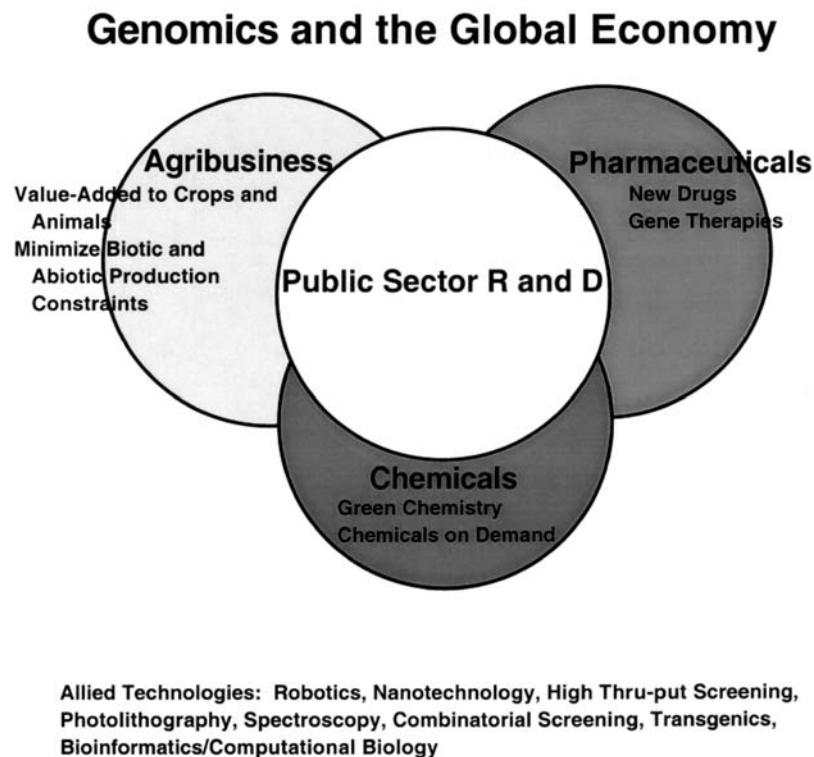


Figure 3 Impact of genomics on the world economy.

2.2.1 Defense Pathways

Despite many projections made in the reviews listed earlier, a flurry of published reports has not followed. In many cases, this can be attributed to the observation being made first in *Arabidopsis* and not in a crop plant. However, this lag is being addressed with *Arabidopsis* genes to assess function in crop plants and through the identification of homologs of *Arabidopsis* genes. For example, the *Arabidopsis NPR1* gene (Cao et al. 1998) when overexpressed in rice caused enhanced resistance to the rice pathogen *Xanthomonas oryzae* pv. *oryzae* (Chern et al. 2001) and the investigators were able to retrieve, in a two hybrid screen, a bZIP family of interactors showing that a similar pathway of signaling is likely present in rice as *Arabidopsis* (Zhang et al. 1999). In addition, Yoshioka et al. (2001) have shown that *Arabidopsis* will respond to the rice fungicide probenazole by induction of PR genes and show enhanced resistance to *Pseudomonas syringae* pv. *tomato* DC3000 and *P. parasitica* Emco5. This response was dependent on a functional *NPR1* gene and was compromised in NahG transgenic plants further supporting the connection of this pathway with generalized resistance to pathogens in both *Arabidopsis* and rice. Six *NPR1* homologs are reported in the recently released Nipponbare genome sequence (Goff et al. 2002). It will be interesting to see how overexpression and silencing of these genes affects resistance of rice to key fungal pathogens of rice such as *Rhizoctonia solani* and *M. grisea*.

Overexpression of the *Arabidopsis ACD2* (accelerated cell death) gene leads to tolerance of susceptible *Arabidopsis* plants to *P. syringae* infection by reducing disease symptoms associated with cell death such as ion leakage, while allowing the bacteria to grow to similar levels as in susceptible plants (Mach et al. 2001). Fungal pathogens were not tested.

Broader testing of other genes that have shown wide-spectrum disease resistance to bacterial, fungal, and viral pathogens when overexpressed such as *Prf* (Oldroyd and Staskawicz 1998) and *Pto* (Tang et al. 1999) in tomato has not been reported. Nor have further reports been made on constitutively active variants of *Pto* (Rathjen et al. 1999). Presumably, this approach can be used in other crop plants.

Introduction of the bacterial blight resistance gene *Xa21* into elite rice cultivars has lead to the expected resistance phenotype when inoculated with *X. oryzae* pv. *oryzae*; however, strains that are virulent on *Xa21* were not tested nor were other pathogens (Tu et al. 1998). Performance of these lines was tested under natural field conditions without any apparent loss of yield performance (Tu et al. 2000).

Disease resistance genes from one crop plant have now been successfully used in other crop species. For example, the *Bs2* resistance gene from pepper confers resistance to *X. campestris* pv. *vesicatoria* in tomato in the laboratory as well as in preliminary field tests (Staskawicz et al. 2002; Tai et al. 1999). Work from my laboratory in conjunction with studies from the laboratories of Mark Farman at the University of Kentucky and Yukio Tosa at Kobe University has suggested that the *Pi-CO39* (*t*) gene (Chauhan et al. 2002)

for resistance in rice to *M. grisea* carrying the *AVR1-CO39* gene (Farman and Leong 1998) will be useful in other grass species such as perennial rye grass as functional copies of the *AVR1-CO39* gene are found in the grey leaf spot pathogen (ML Farman personal communication).

Coexpression of the *C. fulvum Avr9* and tomato *Cf-9* genes in *Brassica napus* was investigated as a method for inducing broad-spectrum resistance to fungal pathogens (Hennin et al. 2001). Induction of *PR1*, *PR2*, and *Cxc750* was detected following injection of the *Avr9* peptide obtained from intercellular fluids of *B. napus* transgenic plants expressing *Avr9* into *B. napus* expressing the tomato *Cf-9* gene. F1 plants and progeny from a cross of the *Avr9* and *Cf-9* plants were evaluated for resistance to fungi. Disease development was delayed at the site of infection of *L. maculans* and *Erysiphe polygoni* but enhanced at the site of infection of *Sclerotinia sclerotiorum*. Thus, heterologous expression of AVR-R gene pairs may be a useful strategy for control of fungal disease in a variety of plants. However, the finding that *Arabidopsis* resistance gene *RPM1* requires another plant gene *RIN4* in order to accumulate and interact with *avrRpm1* or *AvrB* (Mackey et al. 2002) as well as the inability to show direct interaction of the products of *Avr9* with *Cf-9* (van der Hoorn et al. 2002; Luderer et al. 2001) suggests that this strategy must be used cautiously. This may explain the inability of van der Hoorn et al. (2002) to see a necrotic response in the nonsolanaceous plant lettuce with this gene combination. More recent reports on the use of the coexpression strategy for plant protection against fungi in solanaceous plants have not been made (Melchers and Stuver 2000), however the coexpression of *Avr9* and *Cf-9* under control of nematode inducible promoters in tobacco has been studied (Bertioli et al. 2001). Surprisingly these plants underwent spontaneous necrosis in the absence of the nematode. Evidence for activity of the genes was found both in aerial and root tissue.

The cloning of *MLO* locus of barley (Shirasu et al. 1999), which confers nonrace-specific resistance to *Blumeria graminis* f. sp. *hordei*, has been followed with investigations of its potential use for control of different fungal pathogens. Jarosch et al. (1999) found that in contrast to increased resistance conferred by recessive alleles of *MLO* to powdery mildew, these barley plants have increased susceptibility to penetration by *M. grisea* despite showing similar ability to wild type plants to respond to *M. grisea* elicitor. Likewise Kumar et al. (2001) showed that *mlo* plants were more susceptible to the necrotrophic pathogen *Bipolaris sorokiniana*. These reports reveal the complexity of various fungal interactions with the host and the difficulty of using a single strategy to control multiple pathogens.

Recent work on *MLO* has shown that it is a novel calmodulin-binding protein that is responsive to both abiotic and biotic stresses through down regulating the oxidative burst and cell death response (Kim et al. 2002a,b; Piffanelli et al. 2002). Binding to calmodulin is essential to full function of *MLO* (Kim et al. 2002a). A rice homolog of *MLO* that also interacts with calmodulin was isolated (Piffanelli et al. 2002). It will be interesting to see how silencing of *MLO* in rice

plants affects interaction with biotrophic and necrotrophic pathogens.

2.2.2 Antifungal Proteins and Peptides

Several new reports have appeared on the use of antifungal proteins such as Ag-AFP from *Aspergillus giganteus*, chitinase, β -glucanase and Ribosome-Inactivating Proteins (RIP) (Chareonpornwattana et al. 1999; Datta et al. 2002; Oldach et al. 2001), thaumatinlike protein (PR-5) (Datta et al. 1999), and human lysozyme (Takaichi and Oeda 2000) in plants for protection against fungal disease and show different levels of promise for these approaches. Chitinase and AFP appear to increase resistance in wheat, however, the results were not corroborated with levels of these proteins in transgenic plants (Oldach et al. 2001). Other efforts to introduce chitinases in wheat have led to gene silencing (Chareonpornwattana et al. 1999). The recent isolation of cDNA clones for novel acidic chitinases and β -1,3-glucanases from wheat spikes infected by *Fusarium graminearum* (Li et al. 2001) is exciting as these enzymes may be more effective in control of this pathogen in this tissue. Introduction of infection-related chitinase and rice thaumatinlike protein into rice has led to moderate control of sheath blight caused by *R. solani* (Datta et al. 2002). Field evaluation of these plants is underway. Finally, studies on carrot transformed with human lysozyme, which can cleave β -1,4 glycosidic bonds of peptidoglycan in bacterial cell walls and chitin in fungal cell walls, suggest that this approach may have promise for control of *E. heraclei* and *A. dauci* (Takaichi and Oeda 2000).

Natural and synthetic peptides have been evaluated for control of pre and postharvest damage by fungi. Ali and Reddy (2000) studied four cationic peptides for antimicrobial activity *in vitro* and in plants. All were shown to have significant activity in the micromolar range against *P. infestans* and *A. solani* completely inhibiting growth of the fungi on potato tissues. Alfalfa antifungal peptide defensin from seeds of *Medicago sativa* was shown by Gao et al. (2000) to have significant activity against *Verticillium dahliae* *in vitro*, and transgenic potato plants expressing the peptide showed a reduced area under the disease progress curve compared to vector control plants. Moreover, resistance was correlated with the levels of peptide found in root samples.

Similar results were obtained for transgenic potato plants expressing a N terminus-modified cecropin-melittin cationic peptide chimera (Osusky et al. 2000). The efficacy of the peptide against *Phytophthora cactorum* and *F. solani* infection was demonstrated in variety Desiree while not affecting plant growth or tuber morphology or size. Tubers remained resistant for more than one year and the peptide could be detected in this tissue. By contrast transgenic Russet Burbank plants showed significant morphological alterations and resembled lesion mimic plants, produced very small tubers, and showed less resistance to *P. cactorum*. Transgenic raw tubers were fed to mice without significant growth effects relative to untransformed tubers. Rajasekaran et al. (2001)

have shown that the synthetic antimicrobial peptide DE41 is active at the micromolar range against many important bacterial and fungal plant pathogens. Crude protein extracts from transgenic tobacco plants constitutively expressing D4E1 showed ability to reduce growth of *A. flavus* and *V. dahliae* while control plant extracts did not (Cary et al. 2000). Furthermore, the transgenic plants showed increased resistance to *Colletotrichum destructivum*. The D4E1 gene has been introduced into cotton and was shown to be present in cottonseed. Reduction of aflatoxin in cottonseed oil is a desired outcome. Dow AgroSciences LLC has licensed the technology and is collaborating with USDA-ARS scientists who developed the technology to further evaluate the efficacy of the transgenic plants (SeedQuest 2002). The effects of this peptide on plant growth or other nontarget organisms have not been reported. Finally, the antimicrobial peptide MS1-99, an analog of magainin 2, a defense peptide secreted from the skin of the African clawed frog, was expressed from the chloroplast genome of tobacco and showed significant ability to control many phytopathogenic bacteria and fungi (DeGray et al. 2001). Transgenic plant homogenates inhibited the fungi *A. flavus*, *F. moniliforme* and *V. dahliae* and anthracnose lesions were absent in transgenic plant infected with *C. destructivum*. Transformation of the chloroplast genome is an innovative approach to control the spread of the transgene as pollen will not carry the transgenic chloroplast. Evidence for pollen transfer of transgenes at the commercial field level is now available (Reiger et al. 2002).

While the use of peptides has shown significant promise, thorough testing of the toxicity of plants producing these peptides will be important. Their potential ability to inhibit microflora that are essential to plant health as well as health of animals, humans, and birds needs careful evaluation. Feeding raw potatoes containing a cecropin-melittin cationic peptide chimera to mice was not a convincing test of the toxicity as the animals do not normally eat this food and lost weight on this diet until it was supplemented with normal feed (Osusky et al. 2000). More realistic tests are needed. The use of cooked potatoes should be tested. Furthermore, only short-term effects were studied. The survival and biological impact of large quantities of these peptides in the environment resulting from crop plant decay also needs to be evaluated. These issues are only beginning to be addressed in a multitrophic context for insect resistant transgenic plants (Groot and Dicke 2002). These issues need to be critically addressed at the time of risk assessment. Public concern has been spurred largely by a lack on confidence in transgene technology because of the lag time in responding to the large-scale effects that agrochemicals are having on human and environmental health despite early warnings by Carson (1962) many decades ago.

2.2.3 Phytotoxin Detoxification

In planta studies of the hydroxylation and glycosylation of destruxin B, a phytotoxin produced by *A. brassicae*, to a nontoxic product have shown a correlation between plant resistance with phytoalexin production and the efficiency of

these modifications of the toxin in *Brassica* spp. (Pedras et al. 2001). These data suggest that improved resistance can be engineered in or transferred within *Brassica* hosts of *A. brassicae* by enhancing hydroxylation of destruxin B.

The analysis of the biosynthetic pathway of saponins, antimicrobial metabolites of plants, may allow the transfer of these genes to other plants. Mutants defective in the saponin avenacin in oat were studied and shown to define seven loci and to be compromised in their ability to resist fungal attack (Haralampidis et al. 2001). The *sad1* gene was shown to encode β -amyrin synthase.

3 NEW INITIATIVES AND PROSPECTS: THE NEW BIOLOGY

3.1 From Genome Sequence to Gene to Mutant to Function

Research in the last five years has been increasingly driven by the availability of whole genome sequences and cDNA libraries from many plants and microorganisms (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>). The Arabidopsis genome sequence was released in 2001 (Arabidopsis Genome Initiative 2001) and the draft DNA sequence of two subspecies of rice was released this year (Goff et al. 2002; Yu et al. 2002). Likewise, several fungal genome sequences are now available for model organisms as well as plant pathogens (<http://www-genome.wi.mit.edu/annotation/fungi/magnaporthe/>; <http://www-genome.wi.mit.edu/annotation/fungi/neurospora/>; Friedrich et al. 2001; Turgeon and Yoder 2002). Consortia of scientists have been established to bring together fungal genomic resources for a research community. These include *M. grisea* (<http://www.riceblast.org/>) and *Phytophthora* (<http://www.ncgr.org/pgc>; Torto et al. 2002).

Comparative analysis of several fungal genome sequences including that of *C. heterostrophus*, *F. graminearum*, and *Botrytis cinerea*, with those of *Neurospora crassa* and yeast has led to the identification of putative common essential genes, candidate fungal-specific genes, and candidate pathogenicity genes (Turgeon and Yoder 2002). Systematic analysis of these candidate pathogenicity genes through knock out studies is underway. In addition, genes for polyketide synthases and nonribosomal peptide synthetases are being identified and systematically disrupted since many secondary products from fungi are known to have functions as host-specific toxins.

3.2 Random Mutagenesis with Transposons

Another strategy for identification of pathogenicity genes has been to introduce transposons in fungal genomic DNA cloned into cosmid vectors (Hamer et al. 2001). The insertion site of the transposon is sequenced to assess what function has been disrupted. Each tagged insert is then transformed into the

source fungus to create a disruption of the sequence in the genome by gene replacement. This method has been adapted to a high throughput scheme and the resulting mutants are then tested for phenotypic alterations using a battery of tests. The success of this scheme is not dependent on access to the full genome sequence of a fungus.

Agrobacterium tumefaciens T-DNA is also being used to directly mutagenize the genome of a number of fungi (De Groot et al. 1998; Mullins et al. 2001; Rho et al. 2001) as well as plants such as rice (An et al. 2001) and Arabidopsis (see the following; Krysan et al. 1999). *M. grisea* and *F. oxysporum* mutants altered in virulence have been identified (Mullins et al. 2001; Rho et al. 2001). The randomness of insertion in the target genome and the possibility that some mutants may be caused by secondary events needs to be thoroughly evaluated in fungi, nevertheless, this method provides a straightforward way of tagging genes in fungi and appears to be superior to restriction enzyme-mediated insertion (REMI) mutagenesis in which the transforming DNA containing a selectable marker is linearized and transformed with a restriction enzyme to promote insertion at restricted sites in the genome (Balhadere et al. 1999; Bolker et al. 1995; Lu et al. 1994; Sweigard et al. 1998). This method did not generate certain kinds of expected mutants presumably due to the lack of sites for this enzyme in the genes or the inaccessibility of the genes to enzyme because of their lack of expression under the conditions of growth and/or transformation. Mutants lacking a DNA insertion in the mutated gene have also been documented using this method. Thus reisolation of the mutant gene is not simple.

3.3 Transient Expression of Fungal Genes in Plants

Sequenced libraries of cDNAs from *Phytophthora* are being systematically tested for their ability to induce resistance or disease symptoms in tobacco and potato by transient expression using potato virus X (PVX) and *A. tumefaciens* T-DNA vectors as delivery systems (Takken et al. 2000; Torto et al. 2002). Agroinfiltration has been used in several plant systems to study the interaction phenotype of products of avirulence genes and specific resistance genes (van der Hoorn et al. 2000; Tai et al. 1999). Often plants are scored simply for formation of a HR or necrosis. Whether this is truly representative of the natural response to infection is unclear. It will be interesting to see what kinds of the candidate genes are found and whether their disruption or silencing corroborates the heterologous expression tests described here.

3.4 Microarrays

With the recent availability of massive amounts of DNA sequence data for many organisms, new technologies have emerged to study this information in a high throughput and cost effective manner. Microarrays based on spotting of

thousands of cDNAs on glass surfaces or synthesis of gene-specific oligonucleotide microarray (OMA) on glass surfaces have allowed the parallel analysis of expression of large numbers of genes (Brown and Botstein 1999; Fodor 1997; Lashkari et al. 1997). For example, new understanding of: (a) stage-specific gene expression during sporulation in yeast (Chu et al. 1998); (b) the functional role of a set of yeast mutants each carrying a deletion in one of 5,916 open reading frames (96.5% of total) (Giaever et al. 2002); (c) the transcriptional hierarchy of metabolic genes in yeast (DeRisi et al. 1997); and (d) gene expression during meiosis (Primig et al. 2000) has resulted from the use of OMAs. Microarrays are currently being used to study global gene expression in *Arabidopsis* (Zhu et al. 2001a; see later) and rice (Zhu et al. 2001b).

One caution in using these arrays is that they only provide information on gene expression (RNA abundance) while many cellular processes are regulated at the translational or posttranslational levels. Giaever et al. (2002) found that many genes required for fitness through gene knock out did not show increased expression using OMAs. Moreover, many genes that showed increased expression were not required for fitness. In addition, Primig et al. (2000) found very different expression profiles when they compared RNA from different yeast strains undergoing meiosis. The overlapping gene set could be correlated with genes previously known to have a role in meiosis. This result emphasizes the need to use different isolates to find the core set of genes that are needed for a biological process that shows differential gene expression.

3.5 Proteomics

Proteomics is the study of all proteins from a living organism. The most advanced approach is the use of mass spectrometry to study whole cell/tissue protein content and modifications (Haynes and Yates 2000). The method has also been used to analyze proteins in biological complexes (Link et al. 1999). Although this technology is very new and requires sophisticated equipment and technical expertise, it offers a more realistic view of the physiology of the cell at a given point in time. Many studies that have traced RNA expression as a means to identify important genes in a biological process have led to disappointing results when the gene(s) is disrupted (Basse et al. 2000; Giaever et al. 2002; Timberlake and Marshall 1988). Furthermore, redundancy in gene function can confound this type of study. Analysis of the proteome can distinguish which family member is made and modifications that it may have undergone.

Initial studies on the identification of membrane proteins from arbuscular mycorrhizas formed between *M. truncatula* and *Glomus versiforme* led to the identification several protein from microsomes fractionated from mycorrhizal roots using 2D polyacrylamide gel electrophoresis and MALDI-TOF-MS (Mussa et al. 2002). Spectra of the four proteins identified were queried against the EST (expressed

sequence tag) database of *M. truncatula*, and good matches were found with calreticulin, nonseed lectins and an ion channel. These early results are promising, as a homolog of the nonseed lectins is known to be part of the pea nodule where it is thought to function as a storage protein. A similar relationship at this location of the root might exist between the plant and fungus at the arbuscular membrane.

3.6 Plant Model Systems

Considerably more progress has been made toward our understanding of plant genes to date because of the higher investment that has been made in plant biology. From 1985–1995, the Rockefeller Foundation sponsored an international research program in rice biotechnology that led to the generation of rice molecular maps, transformation of rice, and the characterization of many basic biochemical pathways for abiotic stress and disease and insect resistance in rice. The United States National Science Foundation (NSF)-*Arabidopsis* 2010 and Plant Genome Initiatives have also had a major impact on plant science research in the last decade (Ausubel 2002). Many of the projects deal with how *Arabidopsis* responds to and resists pathogens such as: (a) the *Arabidopsis RPM1* disease resistance signaling network; (b) expression profiling of plant disease resistance pathways; (c) functional and comparative genomics of NBS-LRR-encoding genes; (d) Functional genomics of quantitative traits. Expression level polymorphisms (ELPs) of QTLs affecting disease resistance pathways in *Arabidopsis*; and (e) the endgame for research genetics. Isolation and distribution of a knockout mutant for every gene in *Arabidopsis*. More information can be obtained at the web sites of these projects (Ausubel 2002). National Science Foundation Plant Genome has also funded allied work in other crops plants (<http://www.nsf.gov>). This research represents the cutting edge of plant science using the latest tools: microarrays to study gene expression, T-DNA insertion mutants, large scale mutant hunts, molecular mapping, and structure-function analysis of genes at the whole genome level.

High through put methods for creating mutants using gene silencing that allow gene libraries or cDNA collections to be cloned in a silencing vector using an *in vitro* recombinase should facilitate systematic functional analysis of plant genes in plant defense (Wesley et al. 2001). Conventional mutagenesis of plants carrying a construct such as a luciferase or GUS (β -glucuronidase) reporter gene under the control of a defense gene promoter are yielding new and interesting classes of mutants with constitutive broad-spectrum disease resistance (Maleck et al. 2002). These mutants showed strong resistance to *P. parasitica* isolates Noco2 and Emco5 and variable resistance to *Elysiophe cichoracearum*. In addition to T-DNA, endogenous and heterologous plant transposable elements are being used to systematically mutate the rice genome and isolate promoter elements via enhancer trapping elements (Greco et al. 2001; Hirochika et al. 2001). The transcriptome of *Arabidopsis* has been characterized during

systemic acquired resistance (Maleck et al. 2000). Analysis of 402 putative transcription factors from *Arabidopsis* using OMAs allowed the identification of transcription factors that may be involved in regulation of various pathways responsive to environmental stress and bacterial infection (Chen et al. 2002). These systems level approaches to analysis of model plants are expected to yield an integrated view of how every gene contributes to the growth and development and defense of plants.

4 SUMMARY AND CONCLUSION

The tools of biotechnology have already had a significant impact on control of fungal plant pathogens. The use of molecular markers to create genetic maps, identify, transfer, and clone plant genes of importance to plant protection has had a spectacular beginning. With the availability of new types of markers such as cDNA-AFLP and OMAs as well as the genomic and cDNA sequences of many important fungal pathogens and model dicot and monocot plants, rapid progress in this area is anticipated in the near and distant future. These and other genome-wide approaches will lead to an integrated view of the defense response for different classes of fungal pathogens as well as the core of pathogen-specific genes required for successful plant infection of host plants.

This information will lead to new technologies for plant protection based on rational design whether through molecular breeding or transgene or chemical approaches. At present, few promoters related to disease control from plants have been thoroughly characterized. Likewise, our understanding of the rapid turnover of some defense gene products, such as Rpm1, is very poor. What relationship gene transcription has to levels of protein expression and modification and degradation for components of the defense response is unknown. Moreover these relationships must be known at the cellular not tissue level. Knowledge of the complete cascade of players involved defense signaling for one specific pathogen elicitor/effecter is still unavailable. Clearly there is much to learn and much promise ahead based on the knowledge to be gained.

Public confidence in these approaches will require improved communication of the scientific research community with the public sector and comprehensive risk assessment supported by high quality research. Biotechnology is not a solution but a tool to be used along with the many other tools that already exist for plant protection, such as crop rotation and other cultural practices. The appropriate and reasoned use of this technology is needed.

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2

Chemical Identification of Fungi: Metabolite Profiling and Metabolomics

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1 INTRODUCTION

The identification of filamentous fungi has always been considered difficult and many misunderstandings and misidentifications can be found in the literature (Frisvad 1989; Mantle 1987). Phenotypic characters, e.g., morphology and growth on selected media have traditionally formed the basis for fungal taxonomy (Domsch et al. 1980; Mantle 1987; Pitt 1979; Raper and Fennell 1977; Raper and Thom 1949). Advancements in the developments of analytical methodology have allowed the use of “secondary” metabolite profiling for fungal identification and been used to revise the taxonomy within genera of *Penicillium*, *Aspergillus*, *Fusarium*, *Alternaria*, and their perfect states. The success of metabolite profiling in the classification of filamentous fungi relies on the fact that a major part of the fungal growth is expressed by the production of numerous diverse metabolites, most of which are excreted into the media. The extracellular metabolites have been termed *the exome*, a subgroup of the metabolome (all metabolites), and these are related to the genome as illustrated in Figure 1. The reasons why most filamentous fungi produce such a diverse profile of secondary metabolites are still unclear, but they are probably produced as a result of stimuli and are directed against, or support actions on, receptor systems (Christophersen 1996) or as outward directed (extrovert) differentiation products. Possible other functions, include chemical signaling between organisms (Christophersen 1996; Frisvad 1994a). Williams et al. (1989) described their functions as “... *serve the producing organisms by improving their survival fitness*”

Commonly used macro- and micromorphological characters (rough conidia, fluffy mycelia, color, etc.) can be difficult to determine unequivocally and are difficult to link to gene sequences. The production of secondary metabolites can typically be linked to a particular gene sequence or gene cluster and is most likely to be regulated as a response to growth factors. However, there are far more metabolites than genes as demonstrated by modern metabolomics. Schwab (2002) estimates that there might be as many as 10–100 metabolites for each gene in higher organisms. Therefore, a metabolite profile can provide an indirect method of detecting a large set of metabolite coding genes which are expressed at the same time. Metabolite profiling also allows detection of a specific gene cluster through the identification of different compounds from that pathway. Turner (1971) and Turner and Aldridge (1983) suggested subdividing secondary metabolites according to their biosynthetic origin. In this way a selected metabolite originating from a pathway, e.g., polyketide, terpene, diketopiperazine, and cyclopeptide is treated as representative of that particular pathway. This is important, as only a limited number of members of a biosynthetic pathway are expressed under a given set of conditions, e.g., external stimuli and growth conditions (Section 2.2). Mantle (1987) has reviewed secondary metabolite production by *Penicillium* species based on biosynthetic pathways. He suggested a renaming of original isolates according to new taxonomic systems and emphasized that frequent misidentifications have led to errors, especially for isolates no longer available for the scientific community. Frisvad and Filtenborg (1983) first demonstrated the advantage of secondary metabolite profiling in fungal taxonomy with the genus *Penicillium*,

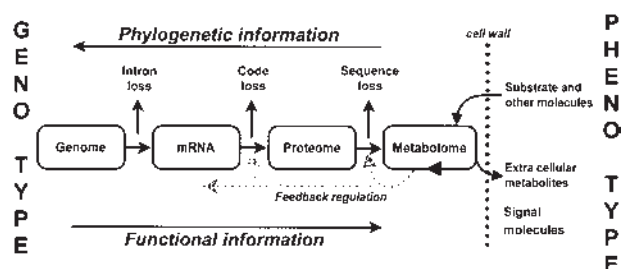


Figure 1 The profile of extra-cellular metabolites or the exome is linked to genomics through a mostly one-way connection from the genome. Far more metabolites than genes are found in most cells, and metabolite profiles are phenotypic characters as are most other characters used in classical mycology.

using a simple agar-plug-TLC technique. They later also included HPLC methods in their studies. Efficient identification based on metabolite profiling relies on combining this information with more classical tools and *a priori* knowledge. For general use it is important to know at least the genus of the fungus being studied and which growth media to use. The analytical methodology depends on the group/genus being studied, but can vary from the simple TLC approach to hyphenated LC-MS-MS. This combined approach is illustrated in Figure 2. It is important to note that efficient identification of fungi will, in most cases, require the use of profiles of metabolites from crude extracts, rather than single or selected metabolites. However, a limited number of species-specific metabolites can be used efficiently as markers for particular species in particular cases. This chapter focuses on the practical considerations in the use of metabolite profiles in identification. The text follows the general approach illustrated in Figure 2, and four different cases of various techniques are given.

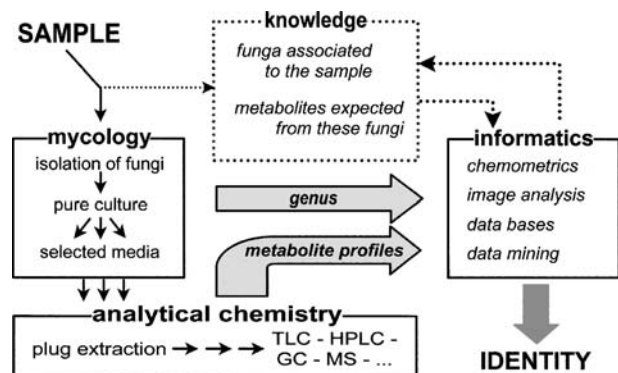


Figure 2 Efficient identification of filamentous fungi requires a synthesis of mycology, analytical chemistry and informatics although the metabolite profile can sometime give the full answer.

2 MYCOLOGY AND BIOSYSTEMATICS

Biosystematics can be divided into taxonomy (=the theory of classification), nomenclature, identification and phylogeny. The species is the central concept in biosystematics. It is important to note that classification deals with the natural nonoverlapping hierarchical grouping of isolates into species, species into genera, etc, whereas identification deals with allocating new isolates to already existing classes in an effective, nonequivocal, and practical way.

2.1 Classical Identification

The characters most often used in identification keys for filamentous fungi are based on arrangements, forms, sizes, and ornamentations of mature asexual and sexual structures. Another kind of latent characters used in identification keys are responses to abiotic factors such as macromorphology and colony growth rate or diameter at different temperatures, water activities, pH, redox potential, etc. (Pitt 1973). The problem with all these different tests is the potential large number needed (economy) and the need for standardization. Morphological features occasionally overlap or can be difficult to record precisely (Frisvad et al. 2000), as they may be dependent on the media used (for example different brands) and is particularly pronounced for colony diameters and colors. Standardization of incubation conditions (Pitt 1973) and the use of chemically defined media have been proposed to avoid these problems. Image analysis has also been explored in *Penicillium* (Dörge et al. 2000), here the information found in fungal colonies such a colony texture, diameter and color from very accurately recorded and calibrated images could identify terverticillate penicillia, and has recently been used for clones recognition (Hansen et al. 2003).

In order to identify filamentous fungi it is necessary to identify the fungi to genus level before using traditional keys or chemotaxonomic methods. Filamentous fungi can be identified to genus level by the use of keys [e.g., Samson et al. (2000)]. Once the genus is known references to keys and taxonomic treatments can be found, but as new species are described each year, it may be difficult to obtain good and up-to-date keys for large genera. Knowledge of the associated fungi (mycobiota) of different habitats can be a major help in identifying the most common species (Filtenborg et al. 1996). For example the only *Penicillia* that can grow on citrus fruits are *Penicillium italicum*, *P. ulaiense*, and *P. digitatum*, so identification of greenish mould growth on citrus fruits will be relatively easy.

2.2 Cultivation and Media for Metabolite Profiling

Most fungi have evolved on solid matrices, and hence solid media are generally better than liquid media in terms of quantity and

number of metabolites produced. On agar media, any contamination is usually visible, and an agar plug technique can be used to sample different parts of the fungal colony and its surroundings.

In general, agar media for optimal secondary metabolite and mycotoxin production have been based on media containing yeast extract. Yeast extract sucrose (YES) broth was introduced as a “semisynthetic” broth medium for aflatoxin production by Davis et al. (1966). It was later shown to be a very effective general secondary metabolite production medium when used with a crude yeast extract (DIFCO or SIGMA) and formulated as an agar medium (YES agar) by Frisvad (1981); Frisvad and Filtenborg (1983), and has been used for *Penicillium*, *Aspergillus*, *Fusarium*, *Alternaria*, and many other fungal genera (Andersen et al. 2002; Thrane 2001). Other media including Czapek yeast autolysate (CYA) agar, Potato dextrose (PD) agar can be used to supplement YES agar, depending on the genus being considered, as seen in Table 1. Some of these semisynthetic agar media can occasionally give problems as certain brands of yeast extract, malt extract, potato extract, agar, peptone, or tryptone, etc. may differ significantly in composition, although this may be diminished by adding trace elements and magnesium sulphate (Filtenborg et al. 1990).

3 TECHNIQUES FOR METABOLITE PROFILING

Metabolite profiling for identification in its simplest form consists of three elements: getting hold of the metabolites (e.g., extraction), determining the compounds (the profile) (e.g., analysis), and data processing (e.g., chemometrics). This will include all relevant metabolites needed for reliable identification of a fungus, and as discussed in the previous

section, profiles of metabolites rather than single metabolites should be used for identification. It is very important to note that metabolite profiles are strongly influenced by the analytical scheme used, thus full metabolite profiles can only be compared if they are produced by the same analytical protocol. Over the years, many specific techniques have been developed to determine a few selected metabolites, mostly known mycotoxins, from cultures and from complex samples such as food and feed. Some of these methods can be expanded to include a broad spectrum of metabolites, but dedicated profiling methods are required to efficiently obtain the best possible metabolite profiles and allow reliable identification.

Developments in chromatography and mass spectrometry (MS) have greatly increased their resolution, sensitivity and productivity in analytical chemistry. There is, therefore, a set of tools available that allows fast metabolite screening from a very small amount of sample. As a result, a broad range of metabolites can now be determined in one analysis with high selectivity. Furthermore, several of the newer techniques have shown their potential as general rapid profiling methodology working directly on raw samples or extracts. These include MS, nuclear magnetic resonance, and FT-IR (including NIR), which eliminate a time consuming chromatographic step. However, currently it is not wise to fully eliminate chromatography and one should rather use systems based on complementary methods. In planning an identification from an analytical approach the following considerations must be made: (a) *A priori* knowledge about metabolites produced by the genus, (b) whether the general chemical classes are alkaloids, acids, neutrals, volatiles, as well as large or small molecules, (c) sample matrix or growth medium composition and interference, (d) number of samples to handle, (e) expected biological variation, and if large or small chemical diversity is expected, (f) sensitivity of analytical instrumentation, and (g) cost.

Table 1 Recommended media for microscopy and chemotaxonomy of important fungal genera

Genus	Teleomorphic state	Microscopy	Metabolite profiling
<i>Penicillium</i> subgenus: <i>Furcatum</i> , <i>Penicillium</i> , <i>Aspergilloides</i>	<i>Eupenicillium</i>	MEA	YES, CYA
<i>Penicillium</i> subgenus <i>Biverticillium</i>	<i>Talaromyces</i>	MEA	OAT, MEA, YES
<i>Aspergillus</i>	<i>Eurotium</i> , <i>Emericella</i> , <i>Neocarpenteles</i> , <i>Neosartorya</i> , <i>Petromyces</i> , <i>Neopetromyces</i> , <i>Chaetosartorya</i> , <i>Sclerocleista</i> , <i>Hemicarpenteles</i> , <i>Fennellia</i>	MEA	YES, CYA
<i>Paecilomyces</i>	<i>Byssochlamys</i>	MEA	YES, PD
<i>Stachybotrys</i>	<i>Melanopsamma</i>	CMA	PD, ALK
<i>Trichoderma</i>	<i>Hypocrea</i>	OAT, CMA	YES, PD
<i>Fusarium</i>	<i>Gibberella</i> , <i>Nectria</i> , <i>Cosmospora</i> , <i>Haematonectria</i>	SNA	YES, PD
<i>Alternaria</i>	<i>Lewia</i>	GAK	DRYES

Media recipes, see text or our website <http://www.biocentrum.dtu.dk/mycology/analysis/>.

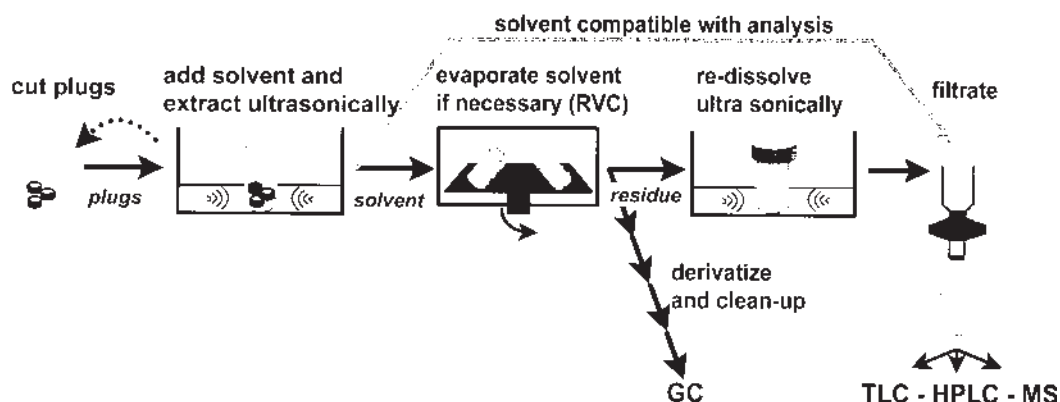


Figure 3 The agar plug extraction method is a simple scheme by which extracts for metabolite profiling can be prepared rapidly using only a minute amount of solvent (Smedsgaard 1997a).

3.1 Extraction

The first step in metabolite profiling for fungal identification is to obtain the metabolites produced by the fungi growing on some sort of substrate, see Section 2.2 and Table 1. Depending on the group of metabolites of interest, two main schemes can be used to collect metabolites for a profile: (a) an extraction approach is used for the nonvolatile metabolites, as illustrated in Figure 3, in combination with HPLC and/or MS and (b) a headspace approach is used for the volatile metabolites as illustrated in Figure 4, in combination with GC or GC–MS.

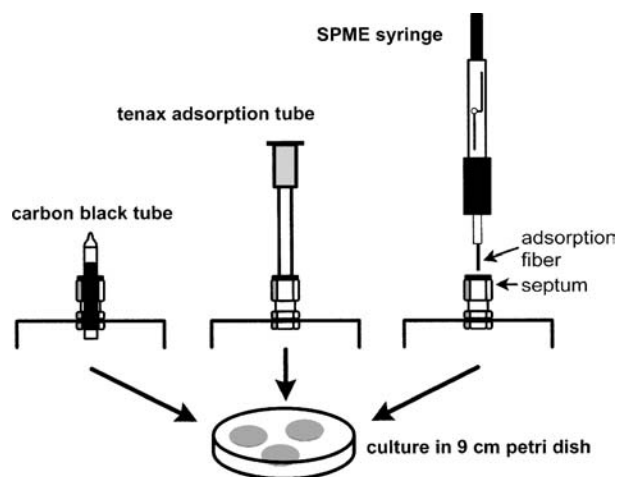


Figure 4 Collection of volatile metabolites using adsorption techniques. A stainless steel petri dish lid with a standard 1/4" Swagelock™ fitting in the centre is used to hold the different adsorption tubes or with a septum to be used with a SPME syringe. Sampling is done by placing an adsorption tube, open only in the lid end, in the fitting and leaving the lid on the cultures for a few hours. The tube or syringe is then analyzed.

Nearly all sample preparations start with either an extraction based on distribution of the analyte between two immiscible phases or by a gas phase sampling. One phase is the fungus (biomass) and/or growth medium, the other is the solvent or a purge gas. Some physical (thermal or mechanical) assistance may be needed to help the rapid distribution of metabolites between the two phases. There may be some overlap in the compounds determined by the two different approaches, e.g., some of the terpenes can be found in both extracts and by headspace analysis. The key point is to select extraction solvents with a high affinity for metabolites of interest. Adjusting pH can enhance the solubility of metabolites in the extraction solvent. Furthermore, choice of extraction solvent can also be used to favor extraction of a particular subgroup, and a selection of several solvents can be used to extend the range of metabolites.

Many extraction protocols use grams of material and many milliliters of solvents, however these procedures can be miniaturized due to the very high sensitivity and selectivity of modern instrumentation. A simple method is the plug extraction method (Smedsgaard 1997a) illustrated in Figure 3, where a few 6 mm plugs (0.5–1 cm² area) are cut from plates of the fungus and extracted with about 500 µl of solvent. The extraction is performed ultrasonically to improve extraction efficiency and speed. If the raw extract is compatible with the subsequent analysis it can be injected directly, otherwise the solvent have to be evaporated and the sample redissolved in an appropriate solvent (Figure 3). Other analytical applications requires a more elaborate sample preparation particularly if GC–MS analysis is required.

If fungi are to be identified in complex natural samples, e.g., foods or building materials a much more elaborate sample preparation protocol is needed. It will nearly always be necessary to remove interfering matrix compounds, by liquid–liquid extraction or by passing the crude extract through a disposable mini-column (solid phase extraction, SPE).

Sampling the volatile metabolites from fungi growing in culture can be done by two approaches: either by one of three different dynamic *headspace* (HS) methods using direct collection of volatiles present in the gas phase above the growing fungus or by *extraction* of volatiles present in the fungal biomass (or sample). Figure 4 illustrates the simple method to collect volatile metabolites by diffusive sampling described by Larsen and Frisvad (1994; 1995c). The volatile metabolites are collected by adsorption on an adsorbent, e.g., activated carbon black, a synthetic polymer like Tenax TA, or recently a coated fiber (Solid Phase Microscale Extraction, SPME). Carbon black tubes are normally desorbed using solvents, e.g., dichloromethane or diethylether. Tenax tubes and SPME fibers are analyzed by thermal desorption, the SPME fibers in a split/splitless injection port, the tubes using thermal desorption equipment.

3.2 HPLC

HPLC is by far the most common analytical technique used for metabolite profiling due to its versatility, relative ease of operation, and the broad spectrum of metabolites that can be determined directly. Basically, HPLC is an integration of a separation and detection system working with separation in a liquid phase.

Metabolite profiling using HPLC is almost always done using gradient elution on reversed phase material (C_8 – C_{18} phase or similar) with polar mobile phase. The most widely used mobile phases are water-acetonitrile or water-methanol containing some modifier, e.g., tri-fluoroacetic acid (TFA). The flow rates used for metabolite profiling depend on the columns, but in general analytical columns with diameters between 2 and 4 mm are used at flow rates from 0.2 to 1 ml/minute. Smaller diameter columns will give a better separation at the cost of lower absolute sensitivity, as a smaller amount of sample can be injected. As described in the introduction to this section identification of fungi from metabolite profiles can be done either by detection of specific metabolites or by using the full chromatogram as a profile. In both cases it is important to keep the analytical conditions as constant as possible. In the first case, the identification of metabolites can be determined from the retention times used together with standards. Possible spectral detection is described later. The effect of small, unavoidable shift, in retention time over time can be reduced by using a series of alkylphenones to calculate a retention index for each peak in the chromatograms (Frisvad and Thrane 1987). If the full profiles are to be used constant analytical conditions will reduce the alignment needed (Nielsen et al. 1998; 1999). In cases of metabolite profiling, detection of compounds eluting from the HPLC column is mostly done by UV detection, fluorescence detection (FLD), or MS. The UV detection accounts for the majority of the applications, however there is currently considerable growth in the use of MS (see section 3.4). In classical HPLC, compounds are detected by measuring the absorbance of the column eluent at a specific

wavelength. The chromatogram [e.g., plot of absorbance vs. time] shows peaks representing the compounds eluting from the column. This technique requires that the compounds have a chromophore that absorbs light at the selected wavelength and that the solvent is transparent (or at least does not have any significant absorption). Fluorescence can be measured with a selected pair of excitation/emission wavelengths in a similar fashion, if the compounds have a fluorophore. Several mycotoxins have distinct fluorophores, whereas others can be derivatized prior to detection.

Identification of fungi can in some cases be achieved by identifying a set of specific metabolites produced, regardless of the analytical method. This requires that relevant metabolites to be identified with high certainty, and usually requires either use of an authentic standard and/or several spectral and chromatographic techniques.

Spectral detection by a UV-spectrometer (DAD or PDA) is commonly used as these techniques greatly enhance the information available by providing structural information about each peak (compound). In HPLC–UV detection, a UV-spectrum is collected at regular intervals in such a way that between 10–20 spectra are collected across a chromatographic peak. The resulting data file is illustrated in Figure 5 and it is important to understand the structure of these files to fully exploit the data of a full HPLC–UV chromatographic matrix. The chromatographic UV-traces at selected wavelengths are present at the time axis and a UV spectrum is present at a specific retention time. The full data matrix can be visualized as a chemical image of the sample and can also be used for fungal identification without identifying the metabolites (Nielsen et al. 1998; 1999; Thrane et al. 2001).

Figure 5 shows that quite different looking chromatograms can be obtained from the same sample by selecting different wavelengths. This feature can be used to enhance the chromatographic resolution and to find specific metabolites, e.g., viomellein is at 18.69 minutes on the 400 nm trace in Figure 5 and overlaps with puberuline at 18.40 minutes on the 220 nm trace. These traces are all profiles of the sample and as such can be used alone or in combination, or to aid specific detection. As quantitative detection is based on Beers law it is necessary to use the same wavelength for quantifying standards and unknowns for each compound but it is not necessary to use the same wavelength for different compounds.

Identification or characterization of compounds can be achieved from UV spectra if the compound has a characteristic chromophore structure (bond structure typical containing π -electrons systems), e.g., xanthomegnin, which is two anthraquinone systems, has a chromophore giving an absorption maximum around 410 nm. This is fortunately the case for many fungal metabolites but not for all (Cole and Cox 1981). The combination of retention time or preferably the retention index and a characteristic UV spectrum is quite reliable for identification of many fungal metabolites (Frisvad and Thrane 1987; 1993), however standards are needed for confirmation. The use of UV-spectra in combination with other detectors, e.g., FLD can greatly enhance the specificity.

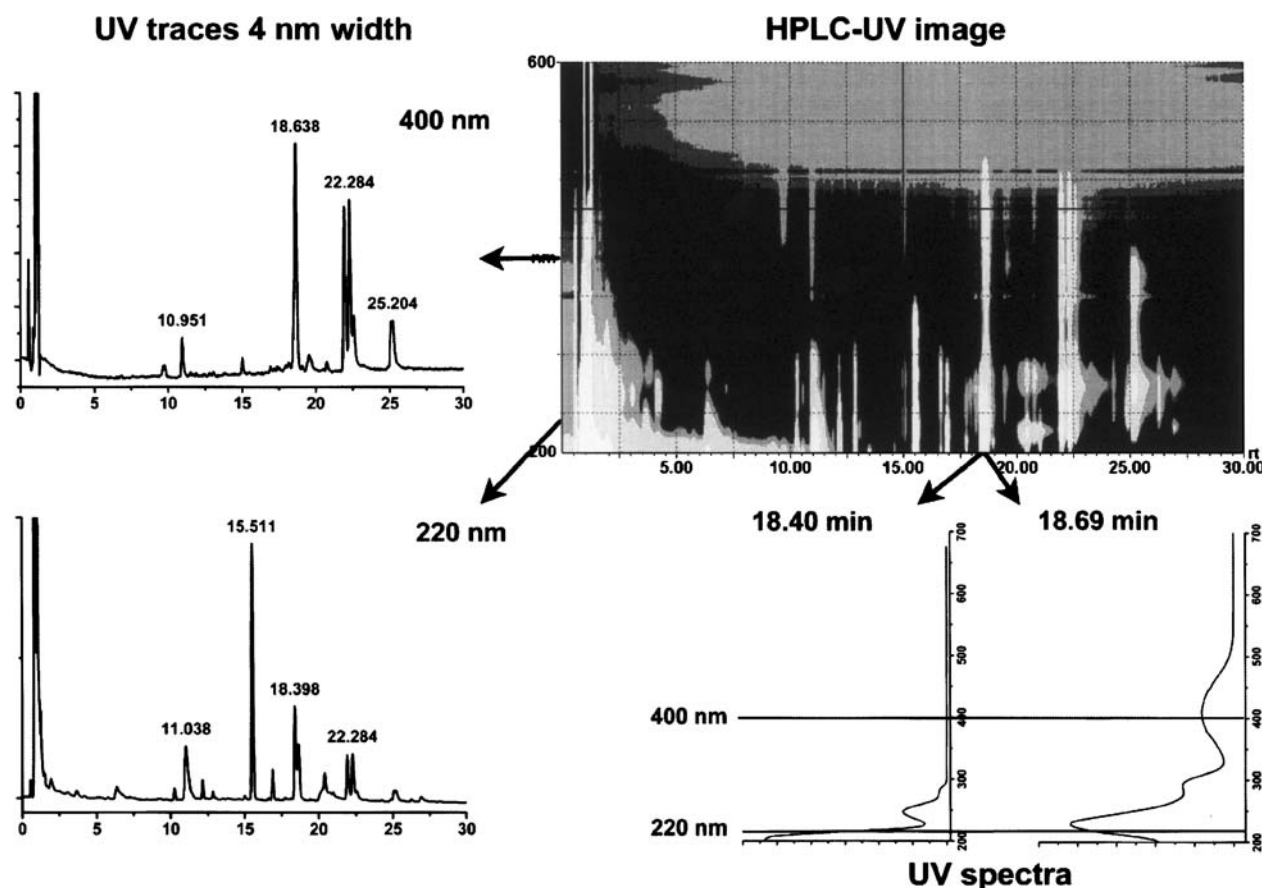


Figure 5 The HPLC-UV data matrix showing both information about composition in form of chromatographic traces and structural information in form of UV spectra. Analysis of a plug extract from *Penicillium cyclopium*, IBT 16932, grown on CYA collecting approx. one spectrum/sec, with a resolution of 4 nm.

For identifying fungi, it is not necessary to know the identity of all of the components in a chromatographic profile. If a peak has unique features in terms of retention time /index and chromophore it can be designated with a code and used along with the metabolites known from a particular species. The most efficient identification can be done if metabolites are grouped in chromophore families (known and unknown compounds), as compounds with similar UV spectra often belong to the same bio-synthetic pathway. Therefore, if just one member of a chromophore family is present it can be used as indication of that particular pathway is active in a fungus.

3.2.1 Case I. Ochratoxin A Determination in *Aspergillus niger*

Ochratoxins can easily be detected in the two penicillia, *P. nordicum* and *P. verrucosum* as well as in *A. ochraceus* and *Petromyces alliaceus* where they are good species markers. They can be detected using HPLC with UV detection with full scan UV spectral confirmation. However, in the *A. niger*

complex, the two ochratoxin producing species *A. niger* (only 6% of the isolates produces ochratoxins) and *A. carbonarius* produce many interfering components that elute across the whole chromatogram (Figure 6) and obscures ochratoxin detection. The FLD gives a very high specificity and sensitivity (1–20 pg on column), with the possibility of obtaining full scan fluorescence spectra. This is illustrated in Figure 6 where an *A. niger* isolate has been analyzed by HPLC with simultaneous UV and FLD, ochratoxins A and B are hidden in the UV chromatograms under peaks of tetracyclic components. The simultaneous detection of all ochratoxin analogues (α , β , A and B) serves as an extra confirmation.

3.3 Gas Chromatography and GC–MS

Gas chromatography (GC) can be used to analyze volatiles directly and numerous semivolatile components after derivatization, e.g., trichothecenes, amino acids, sugars,

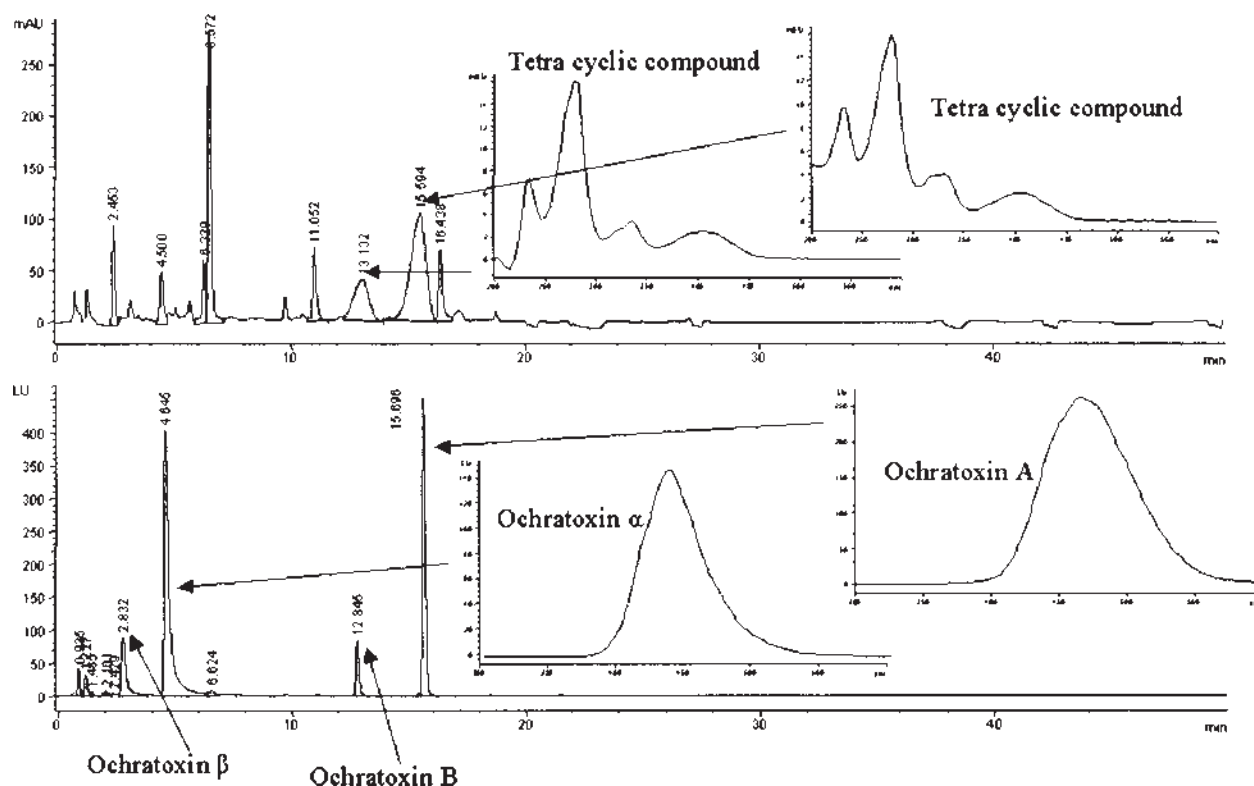


Figure 6 Extract from an *Aspergillus niger* isolate analysed by HPLC-DAD-FLD. Ochratoxin A, B, α , and β , are easily seen in the lower fluorescence trace (ex. 230 nm, em. 450 nm) with the fluorescence spectra of ochratoxin A and α inserted. In the upper UV trace (210 nm), several tetracyclic compounds (UV spectra inserted) obscure the detection of Ochratoxin A, B, and α .

lipids, and sterols. The advantage of GC is its very high separation power in comparison to HPLC and its relative ease of operation. Furthermore, GC is easily interfaced to MS (GC–MS) or other spectral detectors, forming a powerful tool that can both deliver high separation power and give structural information in one run.

The most important step in GC is the injection, which if performed poorly can have a severe effect on the separation power. The most commonly used techniques for liquids are split, splitless, on-column injection, thermal adsorption of trapped volatiles, and headspace (Grob 1993). Currently fused silica columns for gas–liquid chromatography are used due to the high resolution power which is needed to separate complex mixtures of volatiles such as mono- and sesquiterpenes. Different stationary phases as well as film thickness can be chosen depending on the polarity and volatility of the compounds to be separated. More volatile compounds require a thicker film column, whereas high separation power is best obtained by thin film columns (Grob 1993).

The mass spectrometer can be used to: (a) provide structural information from fragmentation in electron impact ionisation (EI) easily searched in databases, (b) *accurate mass* using the modern time-of-flight (TOF) instrument, or (c) using ion-traps (or multistage MS–MS instruments) as very high selectivity detectors (or to get very detailed fragmentation

information). Both TOF and MS–MS instrumentation greatly increases the capability of the instruments, and the metabolite profiles that can be obtained from these instruments are generally not needed for fungal identification and they are therefore not discussed further.

For metabolite profiling, the most important detection method is MS which in many cases will give a molecular ion (and thereby molecular mass) and a characteristic fragmentation pattern—the mass spectrum—from each compound eluting from the column. The limitations of MS in the identification of unknowns can be an insufficient information content of the mass spectrum to stereo and positional isomers in aromatic systems (Ramaswami et al. 1986). A great potential for MS is the ability to scan for a selected number of characteristic ions—selected ion recording (SIR or SIM) which can improve detection sensitivity from the ng level to the pg levels.

Flame ionization detection (FID) is an important and robust GC detector, which basically detects carbon atoms in the sample with high sensitivity and over a large dynamic range, but with no structural information. If metabolite profiles are compared on two different columns of substantially different polarity, similarity in the retention times of metabolites on these two columns can be used for their indirect identification (Davies 1990).

Fourier transform infrared spectrometry (FTIR), can be used to detect compounds eluting from GC columns. Most functional groups, in particular the carbonyl group ($C=O$), have a unique absorption (fingerprint) from vibration energy. The FTIR will therefore give an unambiguous identification of functionality of the functional groups of compounds and it is possible to identify these by comparing spectra to those of known authentic compounds. The method is nondestructive and can therefore be used in combination with either FID or MS. GC-FTIR will often allow discrimination between structural- and stereo-isomers making the method a very powerful supplement to GC-MS. The major drawback of GC-FTIR is a lower sensitivity compared to GC-FID and GC-MS.

3.3.1 Case II: Volatile Metabolites for Identification Of *Penicillia*

Volatile metabolites are believed to play an important role in chemical interactions between fungi and other organisms. Recently the total volatile profile of the endophytic fungus *Muscador albus* was demonstrated to effectively inhibit or kill a number of other fungi and bacteria (Strobel et al. 2001). In general volatile production is correlated with spore formation fitting well with a chemical ecological point of view. The insects, often adapted to the toxic metabolites produced by the fungi, can act as vectors for fungal spores for their further spread.

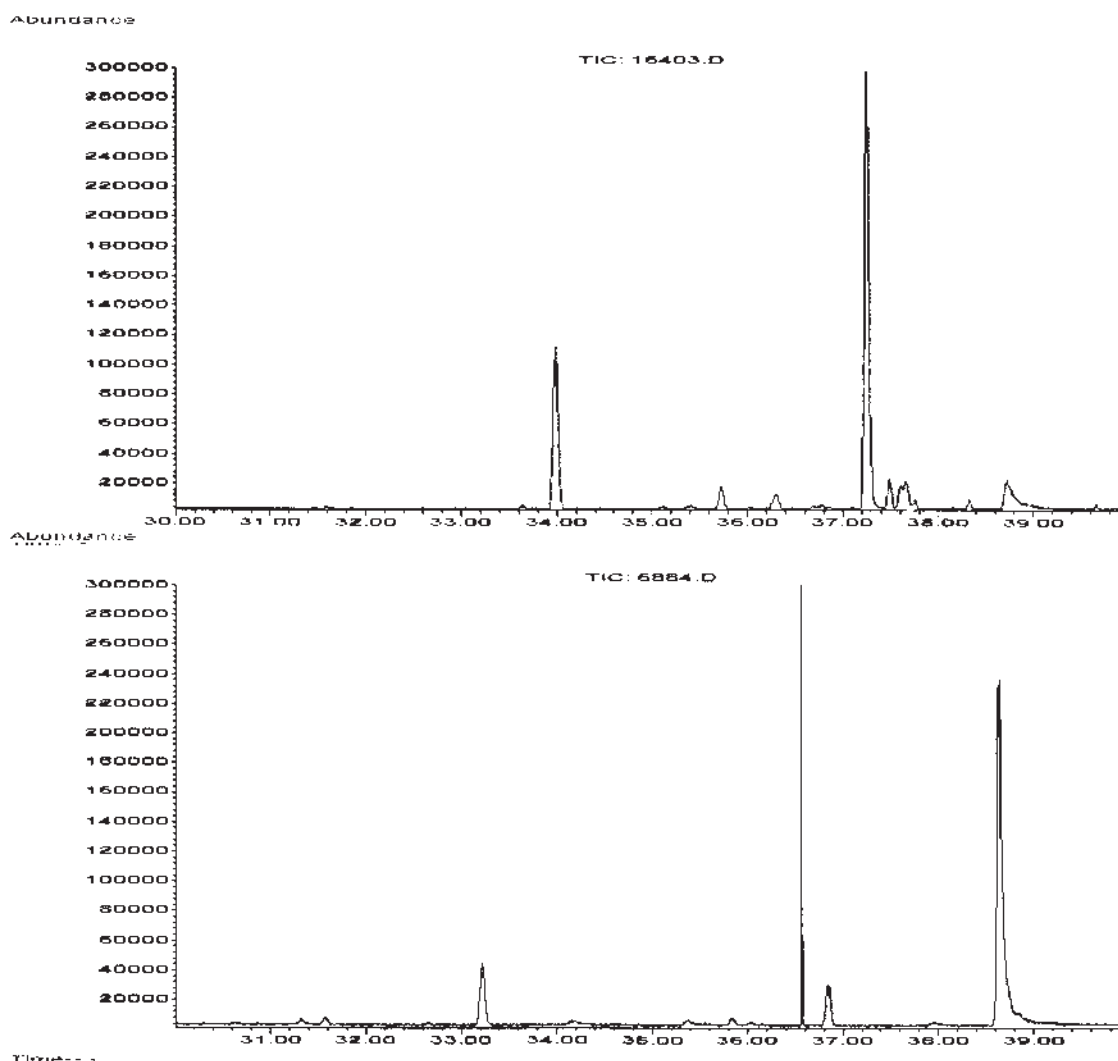


Figure 7 Chromatograms showing profiles of volatile sesquiterpenes produced by *Penicillium roqueforti* (IBT 16403) and *P. carneum* (IBT 6884). The two compounds produced in largest amounts by *P. roqueforti* are β -elemene and (+)-aristolochene, whereas the three largest peaks in the *P. carneum* chromatogram represents geosmin, an unknown sesquiterpene and dodecanoic acid methyl ester.

Some important studies have shown that the production of some possibly species-specific sesquiterpenes could be related to the production of important mycotoxins such as aflatoxins (Zeringue et al. 1993) and trichothecenes (Jelen et al. 1995). Often fungal metabolites are only present in particular compartments of the organism such as in the conidia (or sclerotia), and these may have a role in protecting them against being eaten by other organisms. Microfungi such as the penicillia usually produce a species-specific set of volatile metabolites (Larsen 1998; Larsen and Frisvad 1995a). Often even very closely species such as *P. roqueforti* and *P. carneum* (Figures 7 and 8) can easily be differentiated by the volatiles they produce.

Fungal volatile metabolites include small ketones, alcohols, esters, and hydrocarbons such as small alkenes, mono- and sesquiterpenes. The latter are by far the most relevant metabolites for identification purposes or for use as biomarkers (Larsen and Frisvad 1995b). The major sesquiterpenes produced by *P. roqueforti* are β -elemene, selenine, and patchuline (Larsen and Frisvad 1995b) together with aristolochene (Figure 8), recently reported by Demyttenaere et al. (2002).

In general *P. carneum* produces relatively lower amounts of sesquiterpenes than *P. roqueforti*, however, the species has a much more pronounced moldy odor than *P. roqueforti* due to the production of the moldy smelling compound geosmin together with large amounts of isopentanol (not shown in Figure 8).

It should be emphasized that the production of some volatile compounds is strongly related to medium composition, e.g., lipid degradation of fat rich media leads to the production of ketones and secondary alcohols, as seen in Camembert and especially Blue cheese production. The NIST and Wiley MS databases contains many spectra of mono- and sesquiterpenes (generated a 70 eV) for identification of single

compounds, and a lot of spectral information can also be found in the atlas of Joulain and König (1998). A good review of methods for the identification of sesquiterpenes can be found in König et al. (1999).

As mentioned in Section 3, the sensitivity can be greatly enhanced by the use of SIR, and the method has been used together with SPME to investigate how early volatile metabolites can be detected (Larsen 1997). The SIR of four to seven of the most characteristic ions of mainly sesquiterpenes from cheese-associated fungi allowed the identification to species level within two days, at which time they had not started to sporulate and were only white mycelia. Volatiles from a mixed culture of *P. roqueforti* and *P. commune*, inoculated in a ratio of 1000:1, could be used to detect both fungi within three days, showing the possibility of checking starter cultures for cross-contamination.

3.4 Atmospheric Pressure Ionization MS

The last decade has seen a tremendous development in biological MS, and it is currently one of the fastest growing analytical techniques in biotechnology.

The MS is the determination of the mass to charge ratio of charged species of molecules or highly specific fragments of these (as in GC-MS). These can be either positively charged or negatively charged. In the atmospheric pressure ionization (API) LC-MS techniques, ions are formed at atmospheric pressure and transferred into the vacuum of the mass analyzer. There are two predominant ionization techniques: Electrospray ionization (ESI); and atmospheric pressure chemical ionization (APCI). In ESI the eluant from the column is sprayed through a narrow bore capillary to which a high voltage is applied (around 3 kV). This will produce a spray of highly charged droplets. The solvent is evaporated from the droplets by a heated gas, leading to shrinkage and disintegration to charged species through a complex process. The ions are formed either in the solvent before spraying or during the spray droplet shrinkage and the key parameters influencing the ion production are: solvent composition (surface tension, volatility, modifiers, pH, ion strength), source parameters (temperature, drying gas flow, potential), and interaction between analytes in the sample (Berkel 2000). The charged species are then sampled into the vacuum of the mass analyzer.

In APCI the eluant from the HPLC column is sprayed through a co-axial capillary with a heated gas to evaporate the solvent. Evaporated solvent molecules are ionized by a corona discharge from a needle that is usually placed across the sampling orifice. Analyte molecules are ionized by chemical reaction in the gas phase at atmospheric pressure through a process much like the classical chemical ionization. The ions are sampled into the mass analyzer by a process similar to ESI (see Table 2).

In general, ESI is the most versatile technique for a very broad range of bio-molecules and also the easiest to use, therefore ESI is also the most frequently used technique.

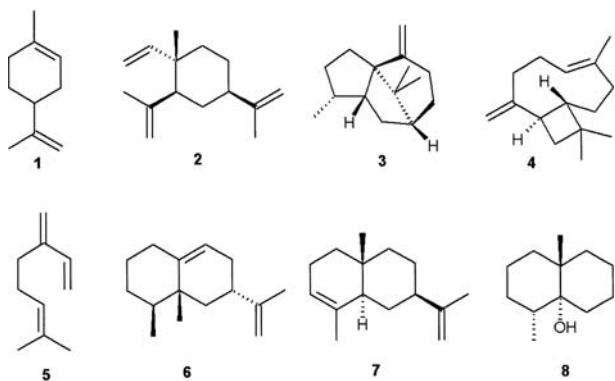


Figure 8 Structures of the main fungal terpene metabolites obtained from the headspaces of *Penicillium roqueforti* and geosmin produced by *P. carneum*. 1) Limonene 2) β -elemene 3) γ -patchulene 4) β -caryophyllene 5) β -myrcene 6) (+)-aristolochene 7) α -selinene 8) geosmin.

Table 2 Comparing some of the characteristics of the two major ionization techniques in LC-MS

	ESI	APCI
Type of ions positive	$M + H^+$, $M + Na^+$, solvent adducts	M^+ , $M + H^+$
Negative ions	$(M - H)^-$	M^- , $(M - H)^-$
Ionization in	Solvent (solvent gas interface)	Gas phase
Solvents	Some ions required	Apolar solvent possible
Fragments	None or few	Common
Multiple charged ions	Yes	No
Clusters and complexes	Many	Few
Mass range	High (multiple charged ions)	As the mass analyser
Typical flow rate with HPLC	5–500 $\mu\text{l}/\text{min}$	300 $\mu\text{l}/\text{min}$ –1.5 ml/min

Not all molecules are ionized (usually protonated) in the positive mode, and are detected much better as negative ions. Gas phase chemistry is however not like solvent chemistry, and many carboxylic acids are much more efficiently protonated in gas phase by positive ESI than determined as anions in negative ESI. Negative ESI will give many fewer adducts and clusters than positive ESI, see Table 3, and so it is therefore easier to interpret spectra. However, a higher sensitivity can be obtained for some classes of compounds in APCI than in ESI.

The charge to mass ratio is determined using a mass analyzer, which is either: Quadrupole, time of flight (TOF), ion-trap, sector (electric and magnetic) or an ion-cyclotron (ICR). These analyzers can be grouped into *scanning* analyzers (mass filters) where ions of just one mass to charge ratio can pass at a time and *nonscanning* analyzers where all ions entering the analyzer are detected (Table 4). Mass resolution and accuracy are the two most important factors for the identification of compounds whereas sensitivity and scan speed are of most chromatographic importance.

Accurate mass determination relies on both sufficient resolution to separate isotopes and a very stable mass to

charge determination. The performance of two common mass analyzers is shown in Figure 9. The TOF analyzer raw data (often called a continuum spectrum) show a resolution of approx 7500 (half height) and the quadrupole approx 900 (half height). Mass spectra are normally used as centroid (stick) spectra for mass determination where the stick is placed at the center of the continuum peak. This also reduces the disk space needed to store the spectra.

Formula can be calculated from the mass, and with sufficient mass accuracy the number of possible structures is limited if sensible limits for composition are applied (Table 5). Combining a mass spectrometer with HPLC will allow the recording of mass spectra as peaks are eluted from the column. As was the case collecting UV-spectra, mass spectra are collected continuously with about 10–20 spectra across a peak to produce a data matrix containing both chromatographic and mass spectral information see Figure 10.

Ion traces are highly specific chromatographic profiles of the samples which depend on the mass accuracy. Figure 10 shows an example of high accuracy ion traces corresponding to the protonated mass of puberulone (444.2287 Da) and xanthomegnin (575.1187 Da) both using a window width of 15 ppm (6.7/8.7 mDa) showing only one peak in each; compare this to Figure 5. For each peak a mass spectrum can be retrieved, given structural information about the sample. In this case using an accurate TOF mass spectrometer also gives an estimate of peak formula. Comparing the HPLC-MS image in Figure 10 to the HPLC-UV image in Figure 5 there are significantly more details and higher specificity in the former of these two. The data shown on Figures 5 and 10 were acquired during the same run using a nondestructive UV detector in series with the MS to give the maximum information.

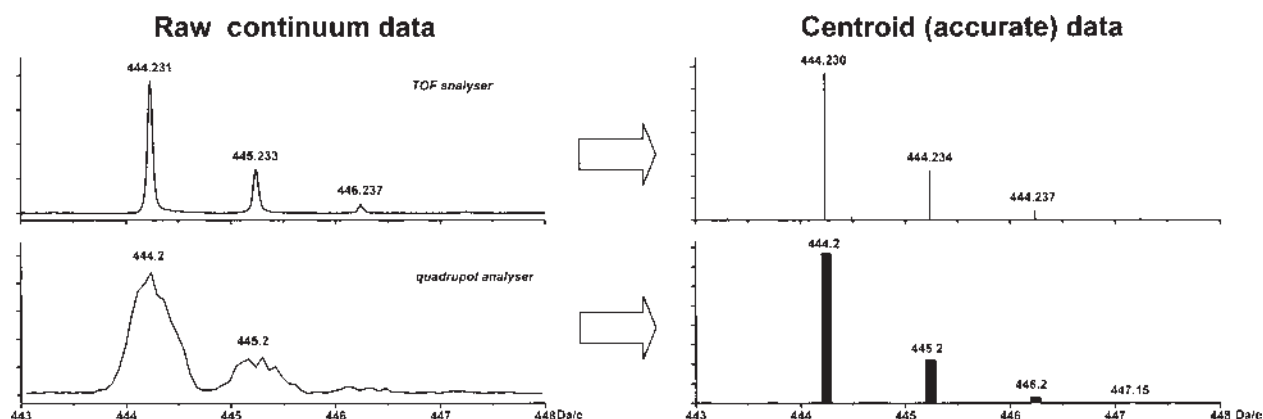
There are some restrictions on the use of HPLC-MS which depend on the ionization techniques: the eluants must be volatile including modifiers (e.g., acids), the flow rate must be suitable for the interface and modifiers/ion strength must match the ionization technique (ESI +/– or APCI +/–). In general, most reversed phase chromatographic solvent systems can be used, e.g., water, acetonitrile, and methanol with the modifiers acetic acid, formic acid, and ammonium

Table 3 Quasi-molecular ions are often seen along with adducts in a low mass positive electrospray mass spectrum. This is highly solvent and parameter dependent. Very few adducts and clusters are seen in negative ESI

	Adducts
Charging by	H^+ , Na^+ , K^+
Solvent adducts	H_2O , CH_3OH , CH_3CN
Dimers	$2M + H^+$, $2M + H_3O^+$, $2M + Na^+$
Multiple charged	Double charged is rarely seen below mass 800–1000 Da/e
Fragments	$-H_2O$,
Others	Fe, Zn, and other metal complexes are sometime seen e.g. $(M - H^+) + Fe^{3+} + H_3CCOO^-$ M^+ ions can be seen from some types of compounds

Table 4 Typical performance of the different mass analyzer although this may vary due to special designs. The sensitivity is relative to the other analyzers and dependent on the analytical approach

	Resolution	Accuracy	Accuracy at 1000 Da	Sensitivity	Scan speed	Relative price
Quadrupol	1000–2000	100–300 ppm	0.1–0.3 Da	medium	high	low
Iontrap	1000	100–300 ppm	0.1–0.3 Da	medium	high	low
TOF	> 10.000	2–5 ppm ^a	2–5 mDa ^a	high	Very high	Medium
Sector	> 50.000	1–5 ppm ^a	1–5 mDa ^a	high	low	High
FT-ICR-MS	> 100.000	0.05–0.1 ppm ^a	0.05–0.1 mDa ^a	high	Very high	Very high

^a Using internal mass correction.**Figure 9** Comparing mass resolution and accuracy of the puberuline M + H⁺ ion C₂₇H₃₀O₃N₃ mass 444.2287 Da/e) from analysis of a crude plug extract see the case below. A peak width at half height of about 0.06 Da and a precision of approx. 1 mDa (centroid data) is found by the TOF instrument (top) and a peak width of about 0.5 Da and a precision of approx. 30 mDa by the quadrupole instrument (bottom).

acetate. However, trifluoroacetic acid should particularly be avoided in APCI (both positive and negative), whereas it can be used in low concentration in positive ESI. It is very difficult to run ESI in pure organic solvent due to volatility, and the limit seems to be around 90–95% acetonitrile-water, however APCI works well in a pure organic solvent.

Metabolite profiling by HPLC–MS (ESI or APCI) are very efficient tools for identification and classification of fungi. As the specificity is very high, rapid chromatographic methods can be used. If a list of expected ions can be made, then the

ion traces corresponding to these can easily be drawn. It is then a simple matter to interpret. In most cases it is not necessary to use high resolution/accurate mass spectrometers for fungal identification. In practical identification, a combination of several metabolites with different retention time is used as a mark for each species thereby limiting the number of misidentifications. However it may be necessary to use more than one ionization technique, as some components are difficult to ionize in positive ESI.

If the goal is to get a full profile of all metabolites produced under specific conditions for metabolomics, then high resolution/accurate mass determination is a major advantage, as it also provides the molecular composition of the ions.

Table 5 Number of structures possible for mass 444.230 Da with different mass accuracies

Accuracy	Number of structures	Composition limits
1 ppm (0.44 mDa)	1	C < 100 H < 500
5 ppm (2.2 mDa)	7	O < 12
50 ppm (22 mDa)	61	N < 10
		DBE < 50

3.4.1 Case III: Direct Infusion MS

a. Metabolite Profiling in Taxonomy Of Penicillium Series Viridicata. An advantage of ESI mass spectrometry is that the analytical conditions can be optimized to limit fragmentation and cluster formation. In the ideal case, only protonated molecules are observed from each compound in a

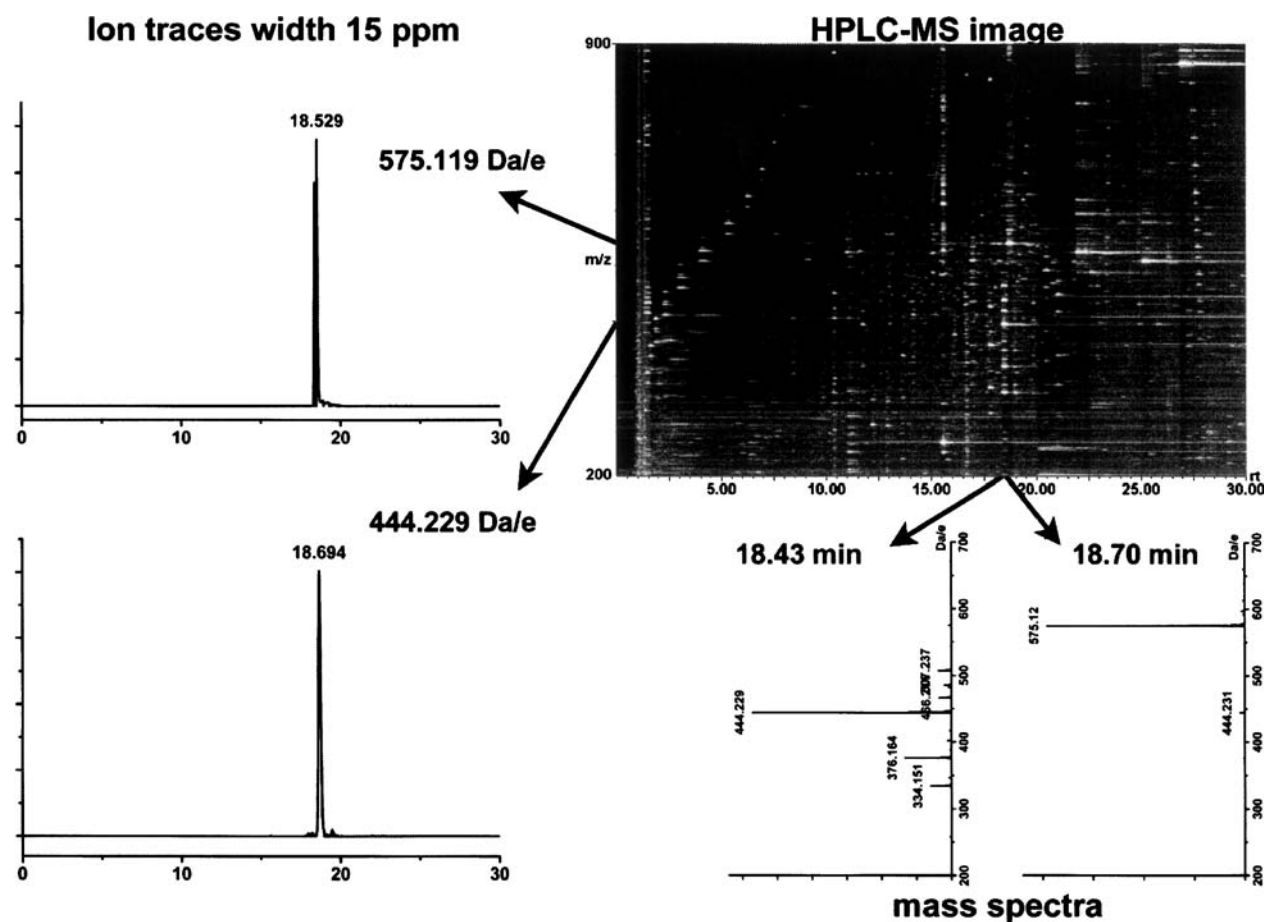


Figure 10 The HPLC-ESIMS data matrix showing both information about composition in form of chromatographic traces and mass information in form of mass spectra. Analysis of a plug extract from *Penicillium cyclopium*, IBT 16932, grown on CYA collecting approx. One spectrum/sec. With a mass resolution of 6000 and an accuracy <5 ppm (Micromass LCT with lockspray).

Table 6 Production of secondary metabolites by cereal associated *Penicillia*

Metabolite	Species ^a								
	I	II	III	IV	V	VI	VII	VIII	IX
Xanthomegnin	0	+	+	0	0	+	+	0 ^b	0
Viomellein	0	+	+	0	0	+	+	0 ^b	0
Aurantiamine	+	+	0	0	0	0	0	0	0
Viridamine	0	0	0	0	0	+	0	0	0
3-methoxyviridicatin	0	+	0	+	+	0	+	0	0
Brevianamide A	0	0	0	0	0	+	0	0	0
Ochratoxin A	0	0	0	0	0	0	0	0	+
Citrinin	0	0	0	0	0	0	0	0	+
Penitrem A	0	0	0	0	0	0	0	+	0
Oxaline	0	0	0	0	0	0	0	+	0
Terrestric acid	+	0	+	0	0	0	0	0	0
Penicillic acid	+	+	0	+	+	+	+	+	0
Verrucosidin	+	0	0	+	0	0	0	+	0

^aI: *Penicillium aurantiogriseum*; II: *P. freii*; III: *P. tricolor*; IV: *P. polonicum*; V: *P. aurantiocandidum*; VI: *P. viridicatum*; VII: *P. cyclopium*; VIII: *P. melanoconidium*; IX: *P. verrucosum* and detected by TLC.

^bThe metabolites can be detected by HPLC.

sample, thus injecting a mixture of compound will give a mass profile of the sample. This approach was used by Smedsgaard in a study of the terverticillate penicillia (Smedsgaard 1997b; Smedsgaard and Frisvad 1997). In that study, crude plug extracts from cultures were injected directly into the mass spectrometer in positive ESI mode with solvent and other parameters optimized to minimize fragmentation and cluster. Samples were used at low concentration to reduce matrix effects, thus avoiding “the winner takes it all effect.” About 10–15 samples can be analyzed per hour by this approach, which have recently been used to study other organisms such as bacteria (Vaidyanathan et al. 2002) and actinomycetes (Higgs et al. 2001).

Nine of the major species in the *Penicillium* series *Viridicata* (The *Penicillium aurantiogriseum* complex) were analyzed by direct injection on a quadrupole mass spectrometer (Smedsgaard and Frisvad 1996). Figure 11 shows crude extract spectra from three different species cultivated on CYA (Samson et al. 2000). The spectra show significant difference between the species and ions corresponding to the protonated mass of major known metabolites. These spectra can be stored and searched in a spectral database using the software included with most instruments, although this software in general is designed for EI spectra (Smedsgaard 1997b). To prove the concept in more detail a cluster analysis of the full centroid mass spectra (normalized) from 45 isolates of the nine major *Penicillium* series *Viridicata* species (Figure 12) was performed. This cluster analysis is done directly on the centroid spectra with 1 Da

resolution using the correlation coefficient and UPGMA linkage.

As it can be seen from Figure 12 most species cluster together, however *P. aurantiocandium* is found in the *P. cyclopium* and *P. tricolor* clusters. One *P. viridicatum* is grouped with *P. aurantiogriseum* and another is an outlier.

The ions (metabolites) that are important to the segregation of the species can be found using Principle Component Analysis (PCA) as the PCA loadings (Figure 13). If we consider loadings above 0.04 or below -0.04 , 47 ions are found in the plot from the first three principal components. Of these, 15 ions correspond to the protonated ions from 15 of the most important metabolites produced by these species (out of about 28 metabolites). Furthermore five ions correspond to the ^{13}C isotope of major metabolites. Five to six characteristic metabolites are not found as they are not produced on these media, however they can be seen in the mass spectra from some of the known producers. Four significant ions of unknown structure are found from the loadings plot at mass 205, 235, 243, and 274 Da.

3.5 A Combined Approach: The Agar Plug-TLC Method

The agar plug method was first introduced as a taxonomic tool for *Penicillium* in 1983 (Frisvad and Filtenborg 1983).

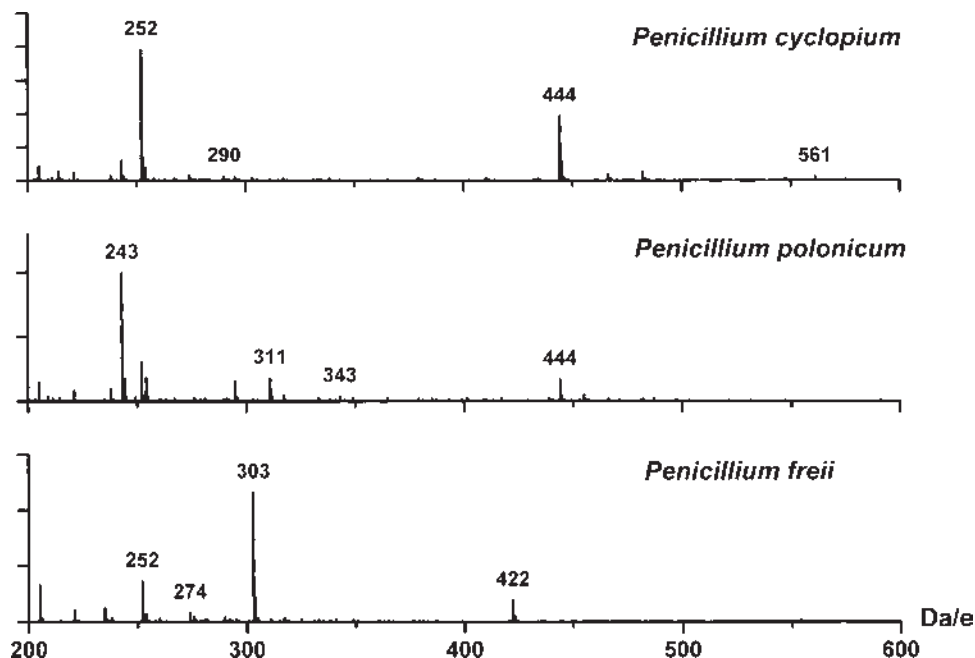


Figure 11 ESI⁺ mass spectra from injection of crude extracts of three *Penicillium* isolates.

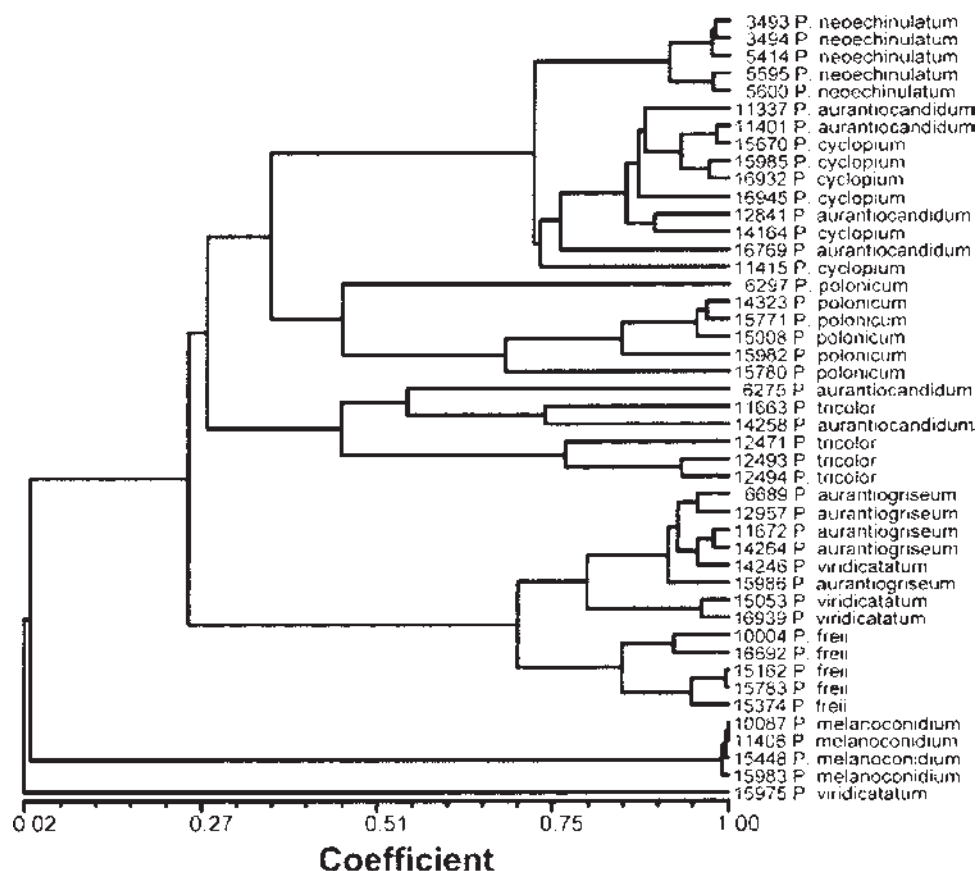


Figure 12 Cluster analysis of 45 mass spectra from direct infusion ESI + MS of crude culture extracts of *Penicillium* species. Calculated using correlation coefficient and UPGMA linkage.

The method uses a combined extraction—TLC analysis, where the extraction is done by adding a drop of solvent on top of a small plug cut from a colony. After a few seconds, the solvent remaining on top of the colony is applied to a TLC

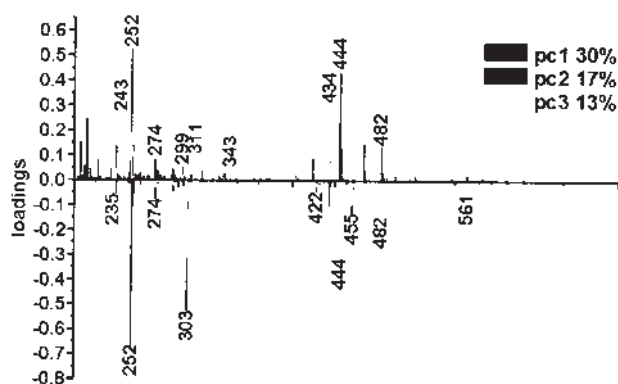


Figure 13 Loadings from the first three principal components from PCA analysis of direct infusion mass spectra collected from 45 isolates of *Penicillium* species from the *Viridicata* series.

plate by gently pressing the wetted side down on the plate. The advantage of the method is that it is simple, cheap equipment is required, sample throughput is high, and with many groups of fungi can be identified as illustrated on *Penicillium* series *Viridicata* (the *P. aurantiogriseum* complex) later. In the general *Penicillium* procedure plugs from CYA media are examined for intracellular metabolites, whereas the plugs from YES are examined for extra-cellular metabolites. A normal 20 by 20 cm plate accommodate 21 lanes from each side, and so 10 isolates can be analyzed (Figure 14).

The TLC plates are eluted in saturated chambers, dried and examined in daylight and under UV light (366 nm and 254 nm) and spots appearing are noted (color, shape, etc.). The spots are marked gently with a pencil (or the plate is photographed). This procedure is repeated after: the whole plate is sprayed with AlCl_3 and heated at 130°C for 8 minutes; the CAP side is sprayed with $\text{Ce}(\text{SO}_4)_2$; the TEF side is sprayed with ANIS and heated at 130°C for 8 minutes.

On our web site <http://www.biocentrum.dtu.dk/mycology/analysis/tlc/> houses a collection of pictures of TLC plates from 18 of the most common *Penicillium* species.

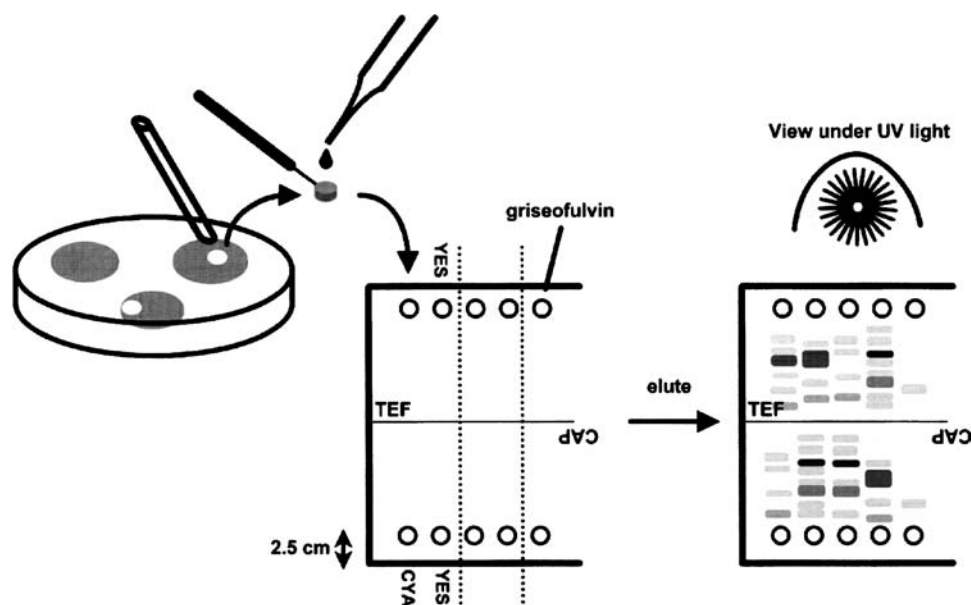


Figure 14 The TLC-agar plug method. A 3 mm plug is cut from a colony and is placed directly on the TLC plate with the agar side down for the extra cellular metabolites or a drop of solvent is added to the mycelium side and the plug is placed with the wetted side down. The plate is eluted in two systems and the dried plate is examined under UV light before and after spraying.

3.5.1 Case IV: Identification of Cereal Borne *Penicillia* by TLC

Cereals represent a habitat with a limited associated fungi, and it is relatively easy to determine *Penicillium* species when kernels are placed on DG18 (Dichloran 18% Glycerol agar), DRYES (Dichloran Rose Bengal Yeast Extract agar) or DYSG (Dichloran Yeast Extract 18% Glycerol agar). When the *Penicillium* species are isolated and inoculated in 3-points on the identification media CYA, MEA (Malt extract agar), YES, and CREA (Creatine Sucrose agar) (Samson et al. 2000) it is relatively easy to observe that on MEA, most isolates have two stage branched rough stipes (terverticillate) and smooth conidia (excluding *P. hordei*), and that growth is inhibited on CREA. At this stage it is not possible to make a definite identification as these criteria fit with the following nine *Penicillium* species found on cereals: *P. aurantiogriseum*, *P. freii*, *P. tricolor*, *P. polonicum*, *P. aurantiocandidum*, *P. viridicatum*, *P. cyclopium*, *P. melanoconidium* (*Penicillium* series *Viridicata*), and *P. verrucosum* (*Penicillium* series *Verrucosa*). Agar plugs from twenty isolates on CYA and YES can be applied on a TLC plate (20 × 20 cm) within 30 min, and the plate developed in TEF (Figure 14).

The two secondary metabolites xanthomegnin and viomellein are always seen simultaneously as two brown spots under visible light in samples from CYA, and reduces the number of possible species to four, i.e. only four cereal-borne *Penicillium* species (Table 6). Aurantiamine, 3-methoxyviridicatin, bre-

vianamide A, viridamine, ochratoxin A, and citrinin are visible as colored spots under UV light (365 nm), usually in highest quantities on CYA. Aurantiamine and viridamine are both seen as blue spots, however viridamine is more light blue and has a lower R_f than aurantiamine. Viridamine will identify the fungus as *P. viridicatum*, and can be confirmed by the presence of brevianamide A as a yellow spot. Aurantiamine can be produced by two species, but simultaneously detection of 3-methoxyviridicatin as a blue spot under 255 nm light, and xanthomegnin and viomellein identifies *P. freii*. *P. verrucosum* is identified by a tailing yellow spot of citrinin and a blue green spot of ochratoxin A. The TLC plate should then be sprayed with $AlCl_3$ and then heated at 130°C for 8 minutes. Penitrem A is the visible as a bluish black spot in daylight and oxaline as a yellow brown spot very close to the application point. If both metabolites are present on CYA the fungus is *P. melanoconidium*. The TLC plate should then be sprayed with ANIS and heated to 130°C for 8 minutes. In cultures from YES yellow tailing spots in daylight identify terrestric acid that in combination with aurantiamine definitely identify the isolate as *P. aurantiogriseum*. A yellow brown spot under UV light on CYA detects verrucosidin, which in combination with 3-methoxyviridicatin identifies *P. polonicum*. *P. tricolor* is identified as producer of terrestric acid, xanthomegnin and viomellein. Except *P. tricolor* and *P. verrucosum* all these species produce penicillic acid seen as a bluish red spot under UV light after ANIS spray. In summary eight of nine species can be identified solely based on TLC results however the species, *P. aurantiocandidum* cannot be

identified using TLC results alone, and must be combined with the poor sporulation on CYA.

3.6 Data Handling, Processing, and Chemometrics

Multivariate statistical methods (chemometrics, taxometrics) are ideal for evaluating chemotaxonomic data. Some of these methods are best used for unsupervised classification approaches such as cluster analysis, multidimensional scaling, correspondence analysis, and PCA, whereas other methods such as Partial Least Squares Discriminant (PLS-D) analysis and soft independent modeling of class analogy (SIMCA) are more suited for discriminant analysis and identification (Frisvad 1994b; Söderström and Frisvad 1984).

The immense quantities of data collected by modern analytic instruments dictates some form of automatic data handling and analysis (Nielsen et al. 1999) although problems such as handling of simultaneous UV, MS, and nuclear magnetic resonance data without component identification needs to be solved, as well as issues relating to the storage in searchable databases, and how data can be combined with other biodiversity information.

4 CONCLUSION AND SUGGESTIONS

Profiles of secondary metabolites provide powerful tools for fungal identification and can give insight in very large parts of the fungal genome, in addition of being functional and ecological characters. Metabolite profiling is currently being revolutionized by developments in MS as well as chemometrics and data handling which is necessary to cope with the immense quantities of data collected (≥ 1 Gb/day). Chemical characters can be used directly in synoptic keys. They are also very suitable for databases as they can be accurately recorded using specific chemical methods. Natural classifications are often based on a polyphasic approach, where ideally many different ecologically relevant characters should be used.

ACKNOWLEDGEMENTS

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3

Isozyme Analysis in Fungal Taxonomy, Genetics, and Population Biology

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1 INTRODUCTION

Isozyme analysis is a powerful technique for assaying genetic variation within and among populations. This versatile approach has been used on bacteria, fungi, plants, and most major groups of animals, from insects and other invertebrates to fish and mammals. The appeal of isozyme analysis stems from its ease of use, low cost, and speed. DNA-based technologies can provide a higher level of polymorphism with concomitantly greater resolution. However, when sufficient variation is present, isozymes provide the fastest, easiest answers to biological questions at a significantly lower cost compared to other available techniques.

Isozyme analysis has a long history in the fungi. Differences among general protein patterns of species of *Neurospora* and *Pythium* were first noted during the early 1960s (Chang et al. 1962; Clare 1963). This approach developed into isozyme analysis with the application of methods for detection of specific enzyme activities. Since the mid 1960s, the technique has been applied to questions of fungal taxonomy, genetics, population biology, and species or strain identification (Micales et al. 1986; 1992) but it is only during the past 15 years that the true potential of the technique has been realized.

The purpose of this chapter is to introduce the concept of isozyme analysis in fungi. Strategies for developing new isozyme systems and for addressing particular questions about fungal taxonomy, genetics, and population biology will be discussed. Particular emphasis will be placed on identifying common pitfalls and barriers to successful implementation of new isozyme systems, especially interpretation of banding patterns, preparation of tissue samples, and methods and approaches to data analysis. Specific

examples of isozyme analysis for the major groups of fungi will be discussed to illustrate the many potential applications of this extremely useful and powerful technique.

2 WHAT IS AN ISOZYME?

Isozymes are different forms of a single enzyme that perform the same or similar function. Each form has some small change that allows it to be distinguished from other forms (isozymes) of the same enzyme. The changes usually result from point mutations in the DNA that cause single amino-acid substitutions into the protein making up the enzyme. Changes that affect the charge or size of the final enzyme may alter its mobility in an electric field. Such changes often can be detected by electrophoresis, and this forms the basis for isozyme analysis.

The terms “isozyme” and “allozyme” often are used interchangeably and can be a source of great confusion. However, the terms are not identical. Isozymes that have been analyzed genetically and are known to be alleles at a single genetic locus can be called allozymes. Therefore, all allozymes are also isozymes, but not all isozymes are allozymes. Isozyme is a more general term and should be used unless the genetic basis of the different enzyme forms is known for certain.

3 ISOZYME TECHNIQUES FOR FUNGI AND OOMYCETES

Many methods have been developed for separating enzyme variants and visualizing isozyme variation. The basic

approach is to obtain crude extracts of total soluble proteins, separate the proteins by electrophoresis through some type of solid matrix (usually a gel), then visualize the isozymes by use of chemicals that produce a visual reaction in response to specific enzyme activity. General methods of isozyme analysis have been described elsewhere (Selander et al. 1971; Shaw and Prasad 1970; Soltis et al. 1983) and will not be repeated here. However, aspects of isozyme analysis that are specific to fungi will be discussed briefly. Additional information can be found in previous reviews of isozyme analysis and compilations of recipes specifically adapted for fungi (Micales et al. 1986).

3.1 Types of Tissue and Preparation of Samples for Isozyme Analysis of Fungi

Fortunately, virtually any type of fungal tissue can be used for isozyme analysis. This could include sections of sporocarps collected in the field or produced in culture, mycelia from plates or liquid cultures, or even conidia washed from lesions or cultures. The main requirement is that the tissue must be living so the enzymes are functional. Old cultures that are dead or dying will not give satisfactory results. This precludes dried herbarium material as the activity of most enzymes will be destroyed during the drying process. However, living material that has been lyophilized and stored at -80°C will maintain enzyme activity for many months and possibly for years. Lyophilization also may be a good option to save material until a sufficient quantity is available for analysis if only limited amounts of material can be collected at a time.

Regardless of source or amount, the fungal material must be macerated to release the enzymes. Lyophilized material can be frozen quickly with a small quantity of liquid nitrogen and ground in a mortar after the nitrogen has evaporated. Small amounts of tissue can be ground in microcentrifuge tubes using a glass rod as a pestle. Disposable plastic pestles designed specifically for use in microcentrifuge tubes also can be used. Fresh tissue can be crushed with a disposable plastic pestle attached to a variable-speed electric drill. Adhering liquid and tissue should be wiped from the pestle between samples, but there is no need to clean the pestle completely as

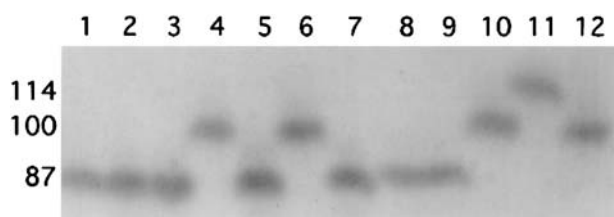


Figure 1 Isozymes of glucose-6-phosphate isomerase in 12 isolates of the barley scald pathogen, *Rhynchosporium secalis*. This haploid fungus yields a single band in each lane corresponding to one of the three putative alleles 87, 100, or 114.

a small amount of cross contamination will not affect the results due to the relatively low sensitivity of the technique. To maintain enzyme activity, all samples should be kept on ice whenever possible. Tissue extracts also can be frozen at -80°C for several weeks if necessary with little or no enzyme degradation. However, this should be tested for each system specifically before used on a large number of samples.

3.2 Choice of Separation Matrix

Gels of 12% hydrolyzed potato starch have been the mainstay of isozyme analysis for decades. Advantages are cost and throughput. A single starch gel can yield 3–4 horizontal slices (possibly more), each of which can be stained for a different enzyme. Depending on the size of the filter-paper wicks used for sample loading and the width of the gel, 20–50 or more samples can be loaded per gel. Thus, more than 200 data points (50 individuals times four enzymes) can be obtained per gel once the system is optimized, and an average of 80 or more is common. This high sample capacity is excellent for population genetics analyses that require large sample sizes for meaningful conclusions. Starch gels can be photographed easily for a permanent record.

A newer approach is to use precast cellulose-acetate plates obtained from commercial suppliers. This technique has been used for many years in medical diagnostics but has only been applied to isozyme analysis in general since the mid 1980s (Hebert and Beaton 1993). Advantages of cellulose acetate are that the pre-cast gels can be purchased ahead of time and stored dry until needed, so there is no time, effort, or skill involved in pouring the gels; specially designed gel boxes and sample applicators can be purchased so a laboratory can be set up quickly; and the complete analysis can be run in an hour from start to finish, so many runs can be completed in the same day. Very small volumes are needed so extremely small amounts of tissue can be analyzed. The stained gels can be photographed or the gels themselves dried and saved for a permanent record.

Isozymes also can be separated on many other types of solid matrix and approach, such as polyacrylamide gels or using isoelectric focusing. Polyacrylamide in particular is often used in an attempt to gain increased resolution. Although these systems often do reveal additional bands, the banding patterns can be difficult to interpret genetically. Furthermore, throughput and ease of use are lower, and costs often are higher compared to starch and cellulose acetate.

3.3 Enzyme Activity Staining

Once the proteins have been separated on a gel, a set of staining protocols must be followed to reveal the isozymes. Specific enzyme activity staining is what makes isozyme analysis possible. Recipes and techniques for staining hundreds of enzyme activities are provided in numerous compilations (Micales et al. 1986; Selander et al. 1971; Shaw

and Prasad 1970; Soltis et al. 1983) and are beyond the purview of this chapter. However, a brief description of the major types of staining systems is provided here. Three staining systems are used commonly for isozyme analysis: positive, negative, and fluorescent. The vast majority of isozyme visualization systems are based on positive staining. The general idea is that activity of a specific enzyme generates a colored precipitate at zones of enzyme activity. These isozymes are visualized as dark bands against a light background (Figure 1). Negatively stained isozymes are visible as light-colored bands against a dark gel. Common negative staining systems include those for the enzymes superoxide dismutase and catalase. A third class of enzyme staining system works by generating bands that fluoresce when exposed to ultraviolet light. Commonly used fluorescent staining systems include those for arylesterase and β -glucosidase.

4 INTERPRETATION OF ISOZYME DATA

The best guide for interpreting isozyme banding patterns is to perform a thorough genetic analysis. Unfortunately, for many fungi this may be difficult or impossible. The purpose of this section is to provide enough information for accurate interpretation of isozyme banding patterns even without a genetic analysis. Accurate interpretation of isozyme banding patterns requires knowledge of the ploidy of the organism and the number of subunits required to form the active enzyme. Most enzymes should give a relatively simple banding pattern with only one to three bands per individual (Figure 1). Enzymes with relatively nonspecific stains (e.g., esterases)

often give complicated banding patterns that are difficult or impossible to interpret without a genetic analysis. Unless crosses can be made easily those systems are best avoided.

4.1 Interpretation of Isozyme Banding Patterns in Haploids

For haploid fungi, each individual ideally will produce a single band on a gel. Such isozymes can be scored easily by assuming that each band corresponds to an allele at a single genetic locus. Each allele can be named by indicating its relative migration distance on the gel. The easiest approach is to designate the most common allele as number 100. Then all other alleles can be indicated by their migration distance relative to the 100 allele. For example, an allele migrating 14% faster than allele 100 would be allele 114, and one migrating 13% slower would be allele 87 (Figure 1).

More than one band per haploid individual may indicate multiple loci. If alleles at each locus migrate within a limited, defined region of the gel then it may be possible to score each locus separately. Alleles at each locus can be named as indicated previously but with a locus designation, e.g., *Gpi-1* 100 and *Gpi-2* 100 to indicate the 100 alleles at two loci coding for the enzyme glucose-6-phosphate isomerase. Enzymes giving more complicated banding patterns in haploids should be avoided as accurate interpretation is almost impossible without thorough genetic analysis. "Null" alleles—those in which no enzyme activity is detected—should be regarded with suspicion as it is difficult to distinguish a null allele from a null caused by faulty technique. Furthermore, it is difficult to imagine a haploid being truly null for important enzyme activities. All potential null alleles should be verified thoroughly—most probably will turn out to be errors caused by poor enzyme extraction from particular individuals or other problems with technique.

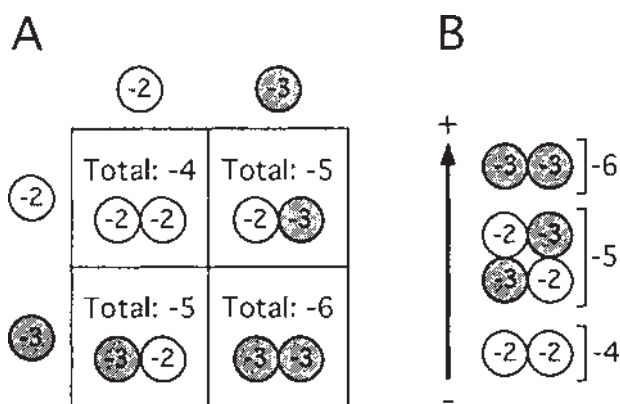


Figure 2 Subunits with -2 and -3 charges combine at random to form an active dimeric enzyme. **A**, In this example, random combination of subunits yields three dimers with total charges of -4 , -5 , and -6 in a ratio of 1:2:1. **B**, The pattern that results on a gel after electrophoresis and staining. Direction of migration is indicated by the arrow.

4.2 Interpretation of Isozyme Banding Patterns in Diploids, Dikaryons, and Polyplids

Interpretation of isozyme banding patterns in diploids and dikaryons is much more complicated, and is aided by knowing whether a particular enzyme is mono- or multimeric. For monomeric enzymes, homozygous diploid individuals should produce a single band on a gel as described for haploids. Diploid individuals that are heterozygous at a locus for a monomeric enzyme will give two bands in a properly stained isozyme gel following electrophoresis. Each band can be interpreted easily as a different allele at a single genetic locus. Banding patterns are more complicated for enzymes composed of two or more subunits. In these cases the subunits must join together to form the active enzyme. Because dimeric enzymes (those composed of two subunits) are the most common, they will be the subject of this discussion. However, the concepts covered here are directly applicable to

enzymes composed of three or more subunits. To correctly interpret isozyme banding patterns produced by dimeric enzymes, it is necessary to understand how the subunits combine to form the active enzyme. In heterozygous diploid individuals, the subunits produced by the two alleles combine at random in the cytoplasm of the cell to form the active molecule. For the hypothetical case of a diploid with two alleles, one coding for a subunit with a -2 charge, the other with a -3 charge, there are four possible ways the subunits can be joined to form the active dimer (Figure 2A). Three types of dimer can result: two homodimers (when identical subunits combine) and a heterodimer (composed of two different subunits). In Figure 2A, each -2 subunit can join with another -2 subunit (to form a $-2/-2$ homodimer) or with a -3 subunit (for a $-2/-3$ heterodimer). Similarly, half of the -3 subunits will pair with a -2 subunit and half with a -3 subunit. Notice that for every $-2/-2$ homodimer (total charge = -4) there are two $-2/-3$ heterodimers (total charge = -5) and one $-3/-3$ homodimer (total charge = -6), for a ratio of 1:2:1.

When these molecules are separated according to charge in an electric field, they will migrate to three regions on the gel, which after staining will be visualized as three distinct bands (Figure 2B). Notice that the middle (heterodimer) band is approximately twice as intense as either homodimer band, reflecting the 1:2:1 ratio in the numbers of each molecule. This pattern is unmistakable on isozyme gels (Figure 3).

The pattern is slightly more complicated for trisomic or polyploid individuals possessing three alleles. In this case, the subunits pair at random as before, only now there are nine possibilities (Figure 4A). If the different alleles vary by single-step charge differences, for example with charges of -2 , -3 , and -4 , then the $-3/-3$ homodimer will have the same -6 charge as the $-2/-4$ heterodimers and will migrate to the same place on the gel during electrophoresis. This will produce a five-banded phenotype in which the intensities of the bands should be in a ratio of approximately 1:2:3:2:1 (Figure 4B). This pattern is seen in the US-8 genotype of the

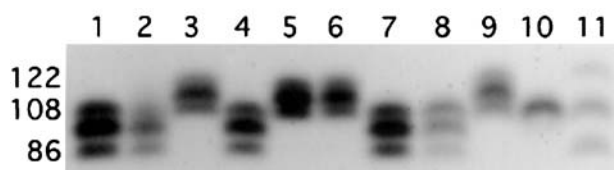


Figure 3 Banding patterns of glucose-6-phosphate isomerase in a diploid. Heterodimers are seen as more intensely staining bands between two other bands. The gel shows interspecific hybrids between the oomycetes *Phytophthora infestans* and its close relative *P. mirabilis*. The *P. infestans* parent was heterozygous 86/122 and the *P. mirabilis* parent was homozygous 108/108. Interspecific hybrids are 86/108 (lanes 1,2,4,7, and 8) or 108/122 (lanes 3,5,6, and 9). Lanes 10 (homozygous 108/108) and 11 (86/122) indicate self fertilization of the *P. mirabilis* and *P. infestans* parents, respectively.

oomycete *Phytophthora infestans* at the Gpi locus, and was confirmed by thorough genetic analyses (Goodwin et al. 1992).

Other banding patterns can be produced by individuals containing three or more alleles separated by unequal charges. For example, an individual heterozygous for three alleles in uneven steps, e.g., -2 , -3 , and -5 , would yield a six-banded pattern in a 1:2:1:2:2:1 ratio. Band-intensity ratios in polyploids also can be affected by the number of copies of particular alleles at a locus. For example, an individual with three alleles, two of which are identical, e.g., -2 , -4 , and -4 , should give rise to a three-banded pattern in a 1:4:4 ratio. This has been documented in the US-1 genotype of *P. infestans* which has two copies of the 100 and one copy of the 86 allele at the Gpi locus (Goodwin et al. 1992). Even more complicated banding patterns are produced by tetrameric enzymes and by multiple loci that share alleles. Interpretation of these patterns was covered in detail elsewhere (Micales et al. 1992). Fortunately, most enzymes are dimeric so the principles discussed previously will apply directly. An understanding of these basic principles combined with genetic analyses should allow unambiguous interpretation of virtually any isozyme pattern.

4.3 Choosing Among the Available Enzyme Systems

Every isozyme project begins with a choice of enzymes. Although it is not possible to predict which enzymes will

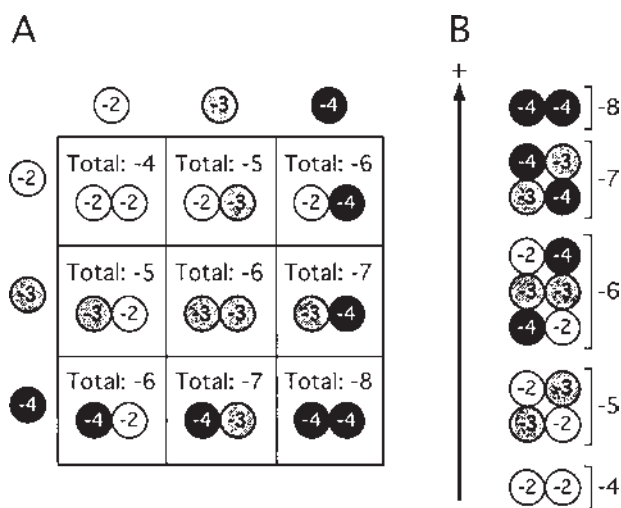


Figure 4 Formation of dimers in a triploid. **A**, The subunits can combine nine ways to form dimers with five possible charges. **B**, Separating these dimers on a gel and staining gives five bands in a ratio of intensities of 1:2:3:2:1. The most intense band results from co-migration of $-2/-4$ and $-3/-3$ heterodimers. All six bands could be visible if the differences in charge among alleles were uneven. Direction of migration is indicated by the arrow.

work with a particular fungal species, some guidance can be obtained from previous research. The best approach is to screen a few isolates on a large number of enzyme systems, then choose those enzymes which give well resolved, easily storable banding patterns. However, choosing which enzymes to try first can be daunting. Enzyme testing results from 27 published surveys plus three unpublished surveys are summarized in Table 1. Among the 82 enzymes listed, 32 never worked and another 20 were successful in less than 50% of the surveys. The most promising enzymes to try in an initial survey would be the 20 enzymes that were tried four or more times and provided useful information in 50% or more of the studies surveyed (indicated in bold in Table 1).

5 APPLICATIONS OF ISOZYME ANALYSIS

Isozyme analysis can be applied to address a wide variety of biological questions in the fungi. These markers usually are considered to be selectively neutral so are ideal for looking at sources of inoculum for pathogenic fungi or gene flow among populations. The markers can indicate whether specific tissues are haploid or diploid, can aid strain or species identification, and can be used to identify hybridization either in the laboratory or in nature.

5.1 Taxonomy and Species Identification

Isozyme analysis was first applied to fungal taxonomy during the 1960s (Clare 1963; Hall 1967; Meyer et al. 1964; Peberdy and Turner 1968). Since then it has been applied at many taxonomic levels from typing individual strains to delimitation and identification of species. The best enzymes for taxonomic purposes are those that are monomorphic within but different among taxa. A caveat is that isozymes cannot be used to infer phylogenetic relationships. This is because bands with the same migration rate on a gel in fact may not be identical. Furthermore, it is not possible to infer which allele is ancestral or to estimate the number of mutations that cause the isozymes to migrate differently; alleles of similar size may be more different evolutionarily than those with larger migration distances on a gel. Therefore, clustering algorithms such as neighbor joining (Saitou and Nei 1987) that allow for unequal rates of evolution on branches are not appropriate for isozyme data. Instead, isozyme data are analyzed usually by calculating a simple distance coefficient and drawing clusters with the Unweighted Pair Group Method with Arithmetic mean (UPGMA) (Michener and Sokal 1957). These analyses can be performed with several computer programs such as NTSYSpc (Rohlf 1998), POPGENE (<http://www.ualberta.ca/~fyeh/index.htm>), PHYLIP (<http://evolution.genetics.washington.edu/phylip.html>), or PAUP* (<http://paup.csit.fsu.edu/index.html>). Cluster analyses always should be accompanied by bootstrap analysis or some alternative method of indicating the level of statistical support for particular groupings. Unfortunately, bootstrap analysis cannot

be performed with NTSYS, but it is available with some of the other computer programs as well as the program WinBoot (Yap and Nelson 1996).

Specific applications of isozyme analysis to taxonomic questions include identifying strains of *Trichoderma harzianum* (Zamir and Chet 1985), varieties of *Verticillium dactylophilum* (Ottosina and Cobb 1987), or anastomosis groups within *Rhizoctonia* (Damaj et al. 1993). The technique also can be used to identify fungal cultures to species (Six and Paine 1997). Isozyme analyses have confirmed a high level of genetic differentiation among host-associated varieties of *Leptographium wagenieri* (Zambino and Harrington 1989) and have revealed previously unknown genetic subdivision within various species of *Phytophthora* (Mchau and Coffey 1994; 1995). In the rust genus *Puccinia*, isozyme variation can distinguish among species and also among *formae speciales* on different hosts (Burdon and Marshall 1981).

The most common use of isozyme analysis in fungal taxonomy is to divide isolates into species and to test how well biochemical species identification corresponds to classical taxonomy. Usually, species groups identified by isozyme analysis correspond quite closely with those identified morphologically (Hsiau and Harrington 1997; Oudemans and Coffey 1991a,b; St. Leger et al. 1992; Surveyer et al. 1995). However, sometimes two or more taxa are found to be the same genetically (Oudemans and Coffey 1991b; Yoon et al. 1990) and are combined into a single species. The opposite also is a common result of isozyme analysis: single species frequently can be divided into two or more species based on previously hidden genetic differentiation uncovered by isozyme analyses (Altomare et al. 1997; St. Leger et al. 1992).

5.2 Genetics

In addition to simple Mendelian genetics (Bonde et al. 1988; Burdon et al. 1986; Hellman and Christ 1991; Shattock et al. 1986a; Spielman et al. 1990) and linkage analysis (May and Royse 1982b), isozymes can be used to distinguish hybrid from nonhybrid progeny in both intra- (May and Royse 1982a; Shattock et al. 1986b) and inter-specific crosses (Goodwin and Fry 1994), and to infer the ploidy level of vegetative hyphae (Goodwin et al. 1994; Shattock et al. 1986b). Dimeric enzymes are ideal for this kind of analysis (Figure 3). Isozymes also can be used to analyze parasexual genetics in fungi. In addition to laboratory genetics, isozyme analysis implicated somatic hybridization as the probable origin of a new *forma specialis* of cereal rust in Australia (Burdon et al. 1981) and identified naturally occurring hybrids among field isolates of *Phytophthora* species (Man in 't Veld et al. 1998). Estimates of relatedness based on isozyme analysis among strains of *Agaricus brunnescens* were used to aid the choice of parents in a mushroom breeding program (Royse and May 1982b). Most studies have shown normal Mendelian segregation of isozyme alleles (i.e., the isozymes can be considered allozymes), although instances of aberrant

Table 1 Isozyme systems tested and success rates in various groups of fungi and oomycetes. The 20 enzymes that were tested four or more times and were useful in 50% or more of the surveys are indicated in bold

Enzyme	E.C. no. ^b	No. times useful/no. times tested ^a				Percent useful	Avg. no. of alleles
		Asco ^c	Basid ^d	Deut ^e	Oom ^f		
Acid phosphatase	3.1.3.2	3/4	6/6	1/3		77	1.6
Aconitate hydratase	4.2.1.3	5/9	3/7	3/5	2/2	56	1.9
Adenosine deaminase	3.5.4.2		0/1		1/1	50	2.0
Adenylate kinase	2.7.4.3	0/3	1/4	0/2	1/1	20	1.0
Alanine aminopeptidase	3.4.11.12		1/3			33	3.0
Alanine dehydrogenase	1.4.1.1	1/1	0/1	0/1		33	1.0
Alcohol dehydrogenase	1.1.1.1	0/4	2/6	0/4	0/1	13	5.0
Aldehyde oxidase	1.2.3.1	0/3		0/2		0	
Alkaline phosphatase	3.1.3.1	2/6	1/5	0/3	0/1	20	2.0
α -Amylase	3.2.1.1			0/1		0	
Aryl (fluorescent) esterase	3.1.1.2	1/1	3/3	1/2	1/1	86	1.8
L-Ascorbate oxidase	1.10.3.3			0/1		0	
Aspartate aminotransferase (glutamate oxalate transaminase)	2.6.1.1	4/7	8/10	3/5	0/1	65	2.3
Aspartate oxidase	1.4.3.16		0/1			0	
Carboxylesterase	3.1.1.1	3/7	4/5	1/4		50	2.6
Catalase	1.11.1.6	1/1	6/7	2/2		90	1.7
Catechol oxidase (tyrosinase)	1.10.3.1			0/1		0	
Creatine kinase	2.7.3.2			0/1		0	
Cytosol (leucine) aminopeptidase	3.4.11.1	7/10	9/14	5/7	1/2	67	2.3
Cytosol nonspecific dipeptidase	3.4.12.18	1/1	1/2	3/5	4/4	75	1.9
Dihydropyrimidine dehydrogenase (diaphorase)	1.8.1.4	2/4	4/8	1/3	1/2	47	2.1
Dipeptidyl carboxypeptidase I	3.4.15.1			0/1		0	
Enolase	4.2.1.1		0/1	1/1		50	3.0
Formate dehydrogenase	1.2.1.2		0/1			0	
Fructose-bisphosphatase	3.1.3.11	0/2	0/2	1/3	1/1	25	2.0
Fructose-bisphosphate aldolase	4.1.2.13	2/2	1/6	0/3	1/2	31	2.5
Fumarate hydratase	4.2.1.2	3/6	2/4	2/5	1/3	44	1.7
Galactose dehydrogenase	1.1.1.48			0/1		0	
β -Galactosidase	3.2.1.23	0/2	0/1	0/1		0	
Glucokinase	2.7.1.2				0/1	0	
Glucose dehydrogenase	1.1.1.47	0/4	0/1	0/1	0/1	0	
Glucose-6-phosphate dehydrogenase	1.1.1.49	7/8	1/5	5/6	2/2	71	1.5
Glucose-6-phosphate isomerase	5.3.1.9	10/10	10/12	5/5	4/4	94	2.3
β -Glucosidase	3.2.1.21	0/1	0/1	2/4	0/1	29	3.5
Glucosyl transferase	2.4.1.11		0/1			0	
β -Glucuronidase	3.2.1.31		0/1	0/1		0	
Glutamate dehydrogenase	1.4.1.2	3/3	5/11	1/3	1/1	56	1.8
Glutamate dehydrogenase NADP	1.4.1.3			1/1		100	1.0
Glutamate pyruvate transaminase	2.6.1.2	1/2	2/3			60	2.0
Glutathione reductase	1.6.4.2	0/1	1/2		0/1	25	2.0
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	0/1	0/4	1/2	0/1	13	2.0
Glyceraldehyde-3-phosphate dehydrogenase (NADP ⁺)	1.2.1.13		0/1	0/1		0	

Glycerate dehydrogenase	1.1.1.29	1/1	0/3	0/1		20	4.0
Glycerol-3-phosphate dehydrogenase (NAD ⁺)	1.1.1.8	0/2	0/1	0/2		0	
Glyoxalase	4.4.1.5			1/1		100	2.0
Guanine deaminase	3.5.4.3			0/1		0	
Hexokinase	2.7.1.1	2/5	4/8	3/4	2/3	55	1.9
Hexosaminidase	3.2.1.30		1/1			100	1.0
3-Hydroxybutyrate dehydrogenase	1.1.1.30	0/1	0/1	0/1		0	
L-Iditol (sorbitol) dehydrogenase	1.1.1.14		0/3	0/1	0/1	0	
Isocitrate dehydrogenase (NADP⁺)	1.1.1.42	5/5	0/5	5/6	2/3	63	1.8
Laccase	1.10.3.2			0/1		0	
L-Lactate dehydrogenase	1.1.1.27	2/5	0/1	0/3	3/3	17	2.0
Leucine dehydrogenase	1.4.1.9		0/1	0/1		0	
Lysine dehydrogenase	1.4.1.15		0/1			0	
Malate dehydrogenase	1.1.1.37	8/9	7/10	5/6	4/4	83	1.9
Malate dehydrogenase (NADP ⁺) (malic enzyme)	1.1.1.40	2/4	3/7	1/4	2/2	47	1.4
Mannitol dehydrogenase	1.1.1.67	3/3	0/2	0/1	0/1	50	1.7
Mannose-6-phosphate isomerase	5.3.1.8	1/3	3/5	2/3	3/3	64	1.6
Menadiene reductase	1.6.99.2	1/1	3/6	2/3	1/2	58	1.1
NAD dehydrogenase	1.6.99.3		0/1			0	
NADPH dehydrogenase	1.6.99.1		0/1			0	
Nucleoside diphosphate kinase	2.7.4.6		1/2	1/1		100	2.0
Nucleoside phosphorylase	2.6.1.1		0/1			50	2.0
Octanol dehydrogenase	1.11.1.7		1/2	0/1		0	
Peroxidase	4.1.1.31		0/2			33	2.0
Phosphoenolpyruvate carboxylase	2.7.1.11			0/1		0	
6-Phosphofructokinase	5.4.2.2	9/9	7/10	5/5	1/2	85	2.3
Phosphoglucumutase	1.1.1.44	6/6	4/6	2/3	4/4	84	1.5
Phosphogluconate dehydrogenase	2.7.2.3		0/3	1/1		25	3.0
Phosphoglycerate kinase	1.14.18.1		0/3			0	
Polyphenol oxidase	1.10.1.1					0	
Pyruvate kinase	2.7.1.40	0/1	0/4	0/2	0/1	9	1.0
Shikimate dehydrogenase	1.1.1.25	1/4	0/3	0/1		0	
Succinate dehydrogenase	1.3.99.1	1/5	0/2	1/3	2/3	31	2.0
Superoxide dismutase	1.15.1.1	0/2	2/2	0/1	1/1	0	
Trehalase	3.2.1.28	2/2	2/2	3/4		89	2.4
Triose-phosphate isomerase	5.3.1.1			1/1		100	1.0
Uridine diphosphoglucose pyrophosphorylase	2.7.7.9			0/1		0	
Uridine monophosphate kinase	2.7.4.4			0/2	1/2	25	1.7
Xanthine dehydrogenase	1.1.1.204	2/5	0/3	0/2		100	4.0
X-Pro dipeptidase	3.4.13.9			1/1			

^a Based on an analysis of 27 published enzyme surveys plus three surveys of septoria pathogens (G. Zhang and S. B. Goodwin, *unpublished*). The published surveys were those of: Altomare et al. (1997); Andrews et al. (1988); Burdon and Roelfs (1985a); Damaj et al. (1993); Gaur et al. (1991); Goodwin et al. (1993); Huss (1996); Leuchtmann and Clay (1989); (1990); Leung and Williams (1986); Linde et al. (1990); Nyassé et al. (1999); Old et al. (1984); Orosina and Cobb (1987); Orosina et al. (1992); Oudemans and Coffey (1991a); Riley et al. (1998); Royse and May (1982a); Six and Paine (1999); Surve-Iyer et al. (1995); Tooley and Fry (1985); Tuskan and Walla (1989); Vogler et al. (1991); Welz et al. (1994); Yoon et al. (1990); Zambino and Harrington (1989); Zhu et al. (1988).

^b Enzyme Commission number. International Union of Biochemistry and Molecular Biology (1992). Enzyme Nomenclature. Academic Press, Inc.

^c Ascomycetes.

^d Basidiomycetes.

^e Deuteromycetes.

segregation have been noted (Spielman et al. 1990). It is important to remember that no isozyme should be referred to as an allozyme until a proper genetic analysis has been completed.

5.3 Population Biology

Gene flow is the movement of individuals among populations and can be estimated indirectly through isozyme analysis as the number of migrants per generation (Slatkin and Barton 1989) or by analysis of “private” alleles—those limited to a single species or population. Different species usually have highly differentiated allele frequencies and a higher number of private alleles compared to individuals from a single panmictic population. The computer program POPGENE, among others, can perform this type of analysis.

Applications of isozyme analysis to epidemiology have included monitoring the spread of specific genotypes of *Phytophthora infestans* (Goodwin et al. 1995; Legard and Fry 1996), determining the influence of various evolutionary forces on the composition of inoculated populations of the barley scald pathogen *Rhynchosporium secalis* (Goodwin et al. 1994), and estimating the size of and boundaries between individuals of the puffball *Lycoperdon pyriforme* on decaying logs (Huss 1993).

The first real attempt to use isozymes to analyze the genetic structure of fungal populations was for *Neurospora intermedia* by Spieth (1975). That analysis revealed a high level of genetic variation within but low differentiation among populations. Spieth’s pioneering work was followed by additional studies within a decade and this has accelerated during the 1990s. Many of these studies have had similar results and demonstrated high gene diversity within but low differentiation among populations of a wide diversity of fungi (Andrews et al. 1988; Goodwin et al. 1993; Huss 1996; Tuskan et al. 1990). Other authors have found varying degrees of subdivision among populations, usually among host-associated forms (Harvey et al. 2001; Leuchtmann and Clay 1989; 1990) and, more rarely, among physiological races (Welz et al. 1994).

Gene flow analysis of isozyme markers also can indicate when taxa have become sufficiently isolated reproductively to be considered separate species. Existence of separate species was shown conclusively for several closely related groups in the genus *Phytophthora* (Goodwin et al. 1999; Man in ‘t Veld et al. 2002; Nygaard et al. 1989) which could not have been discovered without the use of molecular markers. A similar approach identified distinct biological species within the mushroom *Pleurotus eryngii* (Urbanelli et al. 2002).

Isozyme analyses of some populations of fungi and oomycetes have revealed extremely low levels of genetic variation reflective of a highly clonal population structure. This occurred for rice-infecting isolates of the rice blast fungus *Magnaporthe grisea* (Leung and Williams 1986), asexual populations of cereal rusts (Burdon and Roelfs 1985a,b), and worldwide populations of the oomycete

Phytophthora infestans (Spielman et al. 1991; Tooley et al. 1985). Populations of fungi infecting conifer hosts in the western United States also appeared to be highly clonal and specific clones are characterized by fixed heterozygosity (Otrosina et al. 1992; Vogler et al. 1991).

Differences in levels of genetic variation among locations can help identify the center of origin of a species. The most diverse populations genetically should occur at or near the center of origin, as these populations will have had the longest time in which mutations can accumulate; derived populations usually will contain only a subset of the total species diversity. This idea has been combined with isozyme analysis to indicate that the edible mushroom *Agaricus bisporus* may be indigenous to North America (Kerrigan and Ross 1989) and that *Phytophthora infestans*, *P. palmivora*, and *P. megakarya* probably originated in central Mexico (Tooley et al. 1985), southeast Asia (Mchau and Coffey 1994), and central Africa (Nyassé et al. 1999), respectively. Very limited genetic diversity indicated that the plant pathogens *Puccinia graminis* f. sp. *tritici*, *Phytophthora cinnamomea*, and *Dilophospora alopecuri* had only very limited introductions into Australia (Burdon et al. 1982; Old et al. 1984; Riley et al. 1998), although the original source populations for the introductions could not be identified for certain.

6 CONCLUSIONS

Isozyme analysis is an extremely powerful and versatile technique that can answer many questions about fungal genetics, taxonomy, and population biology. Compared to alternative technologies, isozymes are cheap, fast, and easy to interpret. They only assay expressed genes and, by analysis of dimeric enzymes, can distinguish heterozygous individuals from physical mixtures of cultures—an advantage that is not shared by DNA-based markers. With newer types of electrophoresis systems, isozymes are suitable for analysis of small tissue samples and could be portable to remote locations. The main disadvantage of isozyme analysis is a lower level of polymorphism compared to DNA-based markers, but this usually can be overcome by increasing the number of enzyme systems assayed. The many advantages of isozyme analysis should ensure its place in the molecular toolbox for many years to come.

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4

Molecular Methods for Identification of Plant Pathogenic Fungi

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1 INTRODUCTION

1.1 Need for Molecular Methods in Fungal Identification

For centuries, fungal identification has been based on morphological, physiological, and chemical characteristics of specimens. For the most part, these systems still work extremely well. They provide accurate species identification inexpensively, are not labor-intensive, and require little equipment beyond a microscope and chemical reagents. Since phenotype is the result of the expression of hundreds of genes, the higher level classifications based on morphology/physiology are generally sound. A major drawback of the traditional identification methods is that they require some technical training in order to acquire the skills necessary to identify fungi or characterize strains. This training has become increasingly difficult to acquire, whereas molecular biology techniques are now taught widely in secondary schools and can be applied to a multitude of fields. Another drawback of the traditional methods is that they can take a week or more for fungal colonies to grow and develop the characters necessary for identification. In some cases, the necessary characters never develop. Several of the molecular methods provide identifications more quickly and do not rely on the presence of reproductive structures.

Molecular biology has brought many powerful new tools to fungal taxonomists including the potential for rapid identification of isolates, methods for rapid determination of virulence or toxicity of strains, and the means to elucidate the relationships among fungal species. DNA sequence data provide large numbers of data points that can be compared among fungi and analyzed to determine sequence relatedness, which can be assumed to reflect phylogenetic relatedness among species. If “phylogenetic trees” from different

molecular sites concur with one another and with “trees” based on morphological and physiological data, one has fairly strong evidence that the phylogeny is accurate. Some fungi do not grow or do not sporulate in culture. Molecular methodologies allow identification of these isolates by comparison of DNA sequence data from the unknown isolate with sequences from known species. Molecular methods have also been used to distinguish between closely related species with few morphological differences and to distinguish strains (or even specific isolates) within a species. In studies of fungal metabolites, especially mycotoxins, there has been no way of knowing whether a nonproducing strain is truly (genetically) incapable of producing the metabolite or if it could possibly produce it under different environmental conditions. Once the genes of a metabolic pathway have been cloned, they can be used to determine whether or not a strain possesses the genes for production of the metabolite, providing a better indication of the potential of a given strain to produce a metabolite.

1.2 Assumptions and Limitations

The assumptions made in making fungal identifications using molecular methods are the same as those made using traditional methods. The concept of a species (or any other taxonomic level) is ultimately what those working in the field agree it to be. These decisions are based on available data, but there is always some degree of subjectivity in the process, especially when considering closely related species.

None of the current molecular methods used in fungal identification can screen more than a minute portion of the genome. There is no single method that can be used to distinguish all species or all genera from one another. Generally, if the data provided by a molecular method support current taxonomic thought on a taxon, they are used.

A molecular method appropriate for identification of one species or genus (or part thereof) may not be useful for species in other genera or even for all portions of one genus.

Molecular methods provide powerful new tools to aid in fungal identification, but they have not provided (and probably will never provide) a universal solution to problems associated with fungal identification. They are tools, not panaceas, for taxonomists. Currently molecular methods are more labor-intensive and more expensive than many of the traditional methods of fungal identification, but the field is evolving and more rapid and affordable methods will probably become available.

1.3 Scope

It would be beyond the scope of this paper to review all of the studies using molecular methods for identification of plant pathogenic fungi. Instead, we will provide an overview of the methods available for identification and then give one or two recent examples from the literature. These citations will give interested readers a starting point for finding related citations in the literature.

2 METHODOLOGIES

The majority of molecularly-based identification methods rely on the interaction of unique enzymes and nucleic acids. Two extensively utilized classes of enzymes are restriction endonucleases and DNA polymerases.

Restriction endonucleases cleave DNA molecules at specific nucleotide sequences. Different endonucleases recognize and cut at different restriction recognition sequences. An example of two endonucleases and the specific sequence they recognize are *Eco*RI (GAATTC) and *Bam*HI (GGATCC). *Eco*RI cleaves between the guanine and the adenine, G[^]AATTC and *Bam*HI between the guanines G[^]GATCC. Restriction fragment length polymorphism (RFLP) is one molecular technique that requires restriction endonucleases to produce the unique DNA fragments which are the basis for this technique.

Polymerases are enzymes that catalyze the formation of DNA or RNA from nucleotide precursors. All DNA polymerases also require a preexisting template. For example, various DNA polymerases are utilized for different applications [DNA polymerase I (Kornberg polymerase), is used to label DNA; *Taq* DNA polymerase is used to amplify DNA fragments in the Polymerase Chain Reaction (PCR); etc.]; and some have been engineered for enhanced features, e.g., Sequenase[™] which is used in DNA sequencing.

In RFLP the assorted DNA fragments resulting from the restriction endonuclease digestion are resolved by gel electrophoresis. Ethidium bromide staining is then used to reveal the fragments under UV (260 nm) light. Southern blotting of the gel can transfer the DNA fragments to a support membrane. The DNA is then fixed to the membrane

and can be subjected to hybridization analysis. This enables identification of bands with sequence similarity to a labeled probe. Several printed reference laboratory manuals [e.g. Molecular cloning: A Laboratory Manual, 3rd Edition; Short Protocols in Molecular Biology, 4th Edition] and Internet web sites such as www.protocol-online.net and www.nwfs.noaa.gov/protocols detail these techniques. In dot blots, the target nucleic acid sample is affixed directly to the membrane without electrophoresis followed by hybridization with the labeled probe. Detailed information on these protocols can also be obtained by utilizing a search engine such as Google[™] to search the internet.

DNA fingerprinting is an umbrella term that describes molecular identification techniques. As the term implies, the basic genetic material in the organism is utilized to determine its identity and relationship with other isolates. One of the earliest hybridization probes utilized was variable number tandem repeats (VNTRs) or minisatellite DNA (Jeffreys et al. 1985). The term "satellite" originated from buoyant density centrifugation studies in which the bulk of the sample DNA sediments as one main band, but smaller satellite bands were also observed. Satellites are composed of tandem repeats of DNA arranged in consecutive repeats. They sediment apart from the main band containing the bulk of the DNA due to their significantly different base composition. Minisatellites range in size from 1 kb to 20 kb. The VNTR are a type of minisatellite located in the noncoding regions of the genome. The size of the repeat units varies from 9 to 80 base pairs (bp). Smaller repeats consisting of only 1–6 bp are designated microsatellites, or short tandem repeats (STR). The STR have a repetitive region of less than 150 bp. Sequences of both mini- and microsatellites have been utilized in DNA fingerprinting. In Meyer et al. (1991), the DNA of various filamentous fungi were digested separately with different restriction endonucleases and the resulting fragments separated by electrophoresis. Selected VNTR oligonucleotides were labeled with ³²P and hybridized to the dried gel. Results confirmed that the method based on these minisatellite DNA probes could differentiate between species and strains of fungi. Microsatellites have been used to discriminate strains of the aflatoxigenic species *Aspergillus flavus* and *A. parasiticus* (Tran-Dinh and Carter 2000).

The PCR technology provides researchers with a means to rapidly amplify small amounts of DNA, and frees them from the time-consuming task of having to isolate sufficient DNA for RFLP analysis. In PCR a thermostable polymerase is employed to enzymatically amplify a specific region of DNA sequence, defined by a set of two oligonucleotide primers. The target DNA is denatured and the primers are then annealed to the single-stranded DNA. The DNA polymerase then synthesizes the complementary DNA strand across the target region. This process is then repeated and the targeted DNA may be amplified a million-fold or more.

Randomly Amplified Polymorphic DNA (RAPD) is a PCR technique that yields genetic markers without the need to obtain prior nucleotide sequence data (Williams et al. 1990). Random nucleotide sequences are annealed to the template

DNA under low stringency. This is followed by PCR amplification and electrophoresis to produce a DNA fingerprint. The RAPD technique is relatively easy, fast, and requires only a minimum amount of starting material. However, the low stringency of the annealing process can produce PCR artifacts.

Several methods have been developed to reduce the number of artifacts produced by PCR amplification. One method is by using two pairs of PCR primers, or nested primers (Plikaytis et al. 1990). In this technique, the first pair produces a PCR fragment. Then this fragment and the second pair of primers (nested primers) binding to DNA sequence a few bases internal to the first pair of primers, are used in a further amplification. If the first pair of primers amplified the correct locus, then the second pair of primers will produce a slightly smaller PCR fragment. This method requires a knowledge of the sequence immediately adjacent to the first pair of PCR primers in order to synthesize the other "nested" pair of primers.

If the host DNA sequence is not known, Amplified Fragment Length Polymorphism (AFLP) will also reduce the number of PCR artifacts (Vos et al. 1995). In this technique, the target DNA is digested with restriction endonucleases to yield an assortment of different sized DNA fragments. Specific double-stranded adapter oligonucleotides are then ligated to these fragments. PCR primers specific to the adapter sequences with various selective 3' nucleotides are then utilized under high stringency PCR amplification and electrophoresis to produce a unique fragment profile. This technique is time-efficient and amplification is not completely random as in RAPD.

When the sequence of the target DNA is known, several other PCR procedures for identification are available, such as PCR amplification of internal transcribed spacer (ITS) of ribosomal DNA (rDNA). An example of the application of this PCR method is Beck and Ligon (1995) who designed PCR primers to detect *Stagonospora nodorum* and *Septoria tritici* in wheat. These primers were derived from species specific DNA sequences of the ITS of the pathogen's ribosomal DNA. The PCR amplification of ITS rDNA has also been employed to identify a wider variety of fungi that were potential pathogens and allergens (Makimura et al. 2001).

Knowledge of the specific sequence of polymorphic loci permits high-stringency PCR and thus circumvents the problem of artifacts and low reproducibility associated with random-primer methods (Scott and Straus 2000). One method of site-specific polymorphisms they reviewed was based on the sequence variability found in the introns of single copy metabolic and structural genes. Glass and Donaldson (1995) tested several such oligonucleotide primers for their ability to amplify segments of DNA that span introns in a selection of these genes. They identified primer sets that provided a useful tool for phylogenic studies of filamentous ascomycetes and related fungi. These oligonucleotide primers were utilized to differentiate *Fusarium* species (Donaldson et al. 1995). The PCR fragments generated were digested with several 4bp recognition restriction enzymes. The short recognition site,

four bases, of the restriction endonuclease increased the probability that a single base pair polymorphism could be detected.

Two other methods of high-stringency PCR are microsatellite based PCR-amplification and the use of the small subunit ribosomal DNA (SSU rDNA) based primers for DNA fingerprinting (Scott and Straus 2000). For example, Gargas and DePriest (1996) describe a list of PCR primers used to amplify and sequence the small subunit of fungal nuclear rDNA. This information identifies primers for special applications (intron-spanning, intron specific, etc) and represents a valuable resource for further research. Groppe et al. (1995) synthesized oligonucleotides corresponding to regions of the sequence of a microsatellite of the endophytic ascomycete *Epichloë typhina*. These were used for PCR amplification of DNA from different *Epichloë* isolates. The DNA from most isolates produced a single PCR product. This study pointed to a potentially useful role for microsatellite-containing loci as a molecular marker for population studies of *Epichloë* and other unrelated fungi.

DNA sequencing, while not yet extensively utilized because of the time and resources required, has been employed to a limited degree in species identification. Wang et al. (2001) recently reported on the use of mitochondrial cytochrome *b* gene to identify species of *Aspergillus* section *Flavi*. Mycelia of the *Aspergillus* isolates were harvested and their hyphae ruptured with glass beads and zymolyase. Their mitochondria were then collected by centrifugation and their mtDNA extracted. The sequence of the cytochrome *b* gene has also been used to distinguish species of *Aspergillus* section *Fumigati* (Wang et al. 2000) and for investigating the phylogentic relationships of other species of *Aspergillus* (Wang et al. 1998).

Single-Strand Conformation Polymorphism (SSCP) is another PCR-based system that requires knowledge of the target DNA sequence to generate specific oligonucleotide primers. In this technique, the target DNA is concurrently labeled and amplified by PCR using a labeled substrate. The PCR product is then denatured and resolved by electrophoresis. Any changes (mutations, etc.) in the target DNA are detected as altered mobility of separated single strands in autoradiograms. Precise information about the exact change can then be obtained by eluting the targeted DNA from the gel and amplifying it again for sequence determination (Hayashi 1991).

Heteroduplex analysis, like SSCP, is a recently developed technique that can detect a single base difference in target DNA (Keen et al. 1991). PCR amplification products from the isolates are combined after heat denaturation and then allowed to reanneal to form heteroduplexes. Any mismatched nucleotides, caused by substitutions, insertions, deletions, etc, will affect the DNA structure of the heteroduplex and lower its electrophoretic mobility. The heteroduplex is compared to duplexes with complete base complementarity by electrophoresis. Kumeda and Asao (2001) employed this technique for the detection of intraspecific variation in isolates of *Aspergillus* Section *Flavi*. In their heteroduplex panel analysis (HAP), fragments of the internal spacer (ITS)

regions of the rDNA gene of the different isolates were first amplified. Heteroduplexes were then generated with the standard ITS reference fragment and then subjected to electrophoresis. The results of this HAP study corresponded well with the established taxonomy of the Section *Flavi*.

3 RECENT APPLICATIONS IN PLANT PATHOLOGY

Most recent studies in molecular identification have used PCR in some form. This is not surprising given the power of this tool for analyzing DNA. As the use of PCR methodologies in plant disease diagnosis was reviewed by Henson and French (1993), Mills (1996), our emphasis will be on studies conducted since these reviews. The number of studies on various plant pathogenic genera generally reflects the relative importance of these genera in plant pathology. One of the genera receiving the most attention from molecular biologists, for both understanding phylogeny and pathogenicity, has been *Fusarium*. Recent work on this genus has been reviewed elsewhere (Nicholson 2001).

Applications of molecular techniques in plant pathology have provided methods for identification of isolates of plant pathogens, and identification of pathogens directly from plant materials such as leaves, seeds, or roots. These procedures may be applied at almost any taxonomic level, but usually address taxa at species level and below [e.g. races of a given pathogen]. Molecular methods have also proved useful for distinguishing nontaxonomic categories such as virulence or toxicity.

3.1 Identification of Pathogens *In Vitro*

Information from some recently published studies using molecular methods to identify plant pathogenic fungi from

culture is summarized in Table 1. Many of these studies were designed to develop methods to distinguish between closely related taxa within a single genus or species for which morphological or physiological characters overlap or take too long to develop.

There are several advantages in isolating the fungus of interest in culture before conducting molecular identifications. First, one knows immediately whether or not the pathogen is viable in the plant. Second, working with pure cultures lessens the possibility of errors such as accidentally creating a PCR product from the wrong fungus. There are also disadvantages to this approach. First, it takes longer time because the pathogen must be isolated before it is analyzed. Second, some true (obligate) pathogens cannot be cultured in the laboratory. Finally, metabolites such as mycotoxins may remain in the crop even after the fungus dies and these may be missed if no viable fungus is present.

3.2 Identification of Pathogens Directly from Plant Parts

Examples of studies using molecular methods to identify pathogens directly from plant parts are given in Table 2. Most of these involve identification of fungal species using RAPDs or PCR of the ITS-rDNA.

Developing methods for direct isolation of specific fungal DNA from plant tissues is more difficult than isolating DNA from a pure fungal strain, but the potential impact of the former methods is tremendous. These assays have demonstrated the presence of the pathogens in asymptomatic plants (Doohan et al. 1998). Some of these procedures take only seven to 24 h to perform (Lee et al. 2001; Lovic et al. 1995), compared with several days to a week for traditional methods or methods requiring that the fungus be isolated prior to DNA

Table 1 Examples of studies on molecular identification of fungal plant pathogens *in vitro*

Fungal genus	Host	Level of discrimination	Method	Citation
<i>Alternaria</i>	Umbellifers	Species	RAPD	Pryor and Gilbertson 2002
<i>Botrytis</i>	Onion	Species subgroup	PCR-ITS/rDNA	Nielsen et al. 2001
<i>Claviceps</i>	Sorghum	Species/populations	AFLP and RAM	Tooley et al. 2000
<i>Colletotrichum</i>	Alfalfa	Species	AFLP	O'Neill et al. 1997
<i>Elsinoe</i>	Citrus	Species	RAPD	Hyun et al. 2001
<i>Fusarium</i>	Tomato	Virulence within race	RAPD	Mes et al. 1999
<i>Fusarium</i>	Cucumber	Forma specialis	RAPD	Vakalounakis and Fragkiadakis 1999
<i>Gaeumannomyces</i>	Turf-grass	Variety	PCR-ITS/rDNA	Goodwin et al. 1995
<i>Gibberella (Fusarium)</i>	Banana/corn	Species/toxicity/host	RAPD	Jimenez et al. 2000
<i>Gibberella (Fusarium)</i>	Banana/corn	Species	PCR-ITS/rDNA	Jimenez et al. 2000
<i>Macrophomina</i>	Bean, corn+	Population	AFLP	Mayek-Perez et al. 2001
<i>Rhizoctonia</i>	Various	Anastomosis grp subsets	PCR-ITS/rDNA	Carling et al. 2002
<i>Rhynchosporium</i>	Barley	Species	PCR-ITS/rDNA	Lee et al. 2001
<i>Tilletia</i>	Wheat	Species	TaqManPCR-MtDNA	Frederick et al. 2000
<i>Venturia</i>	Pear	Species	PCR-ITS/rDNA	Le Cam et al. 2002

Table 2 Examples of studies on molecular identification of fungal plant pathogens *in vivo*

Fungal genus	Host	Method	Level of discrimination	Citation
<i>Alternaria</i>	Carrot	PCR-ITS/rDNA	Species	Konstantinova et al. 2002
<i>Fusarium</i>	Wheat	RAPD	Species	Parry and Nicholson 1996
<i>Fusarium</i>	Wheat	RAPD	Species/variety	Doohan et al. 1998
<i>Leptosphaeria</i>	Crucifers	PCR/GenBank M77515 ^a	Virulence	Taylor 1993
<i>Melampsora</i>	Willow	RAPD	Stem/leaf variants	Pei et al. 1997
<i>Monosporascus</i>	Cucurbits	PCR-ITS/rDNA	Species	Lovic et al. 1995
<i>Monilinia</i>	Stone fruits	dot blots and PCR	Species	Boehm et al. 2001
<i>Mycosphaerella</i>	Banana/plantain	PCR-ITS/rDNA	Species	Johanson and Jeger (1993)
<i>Peronosclerospora</i>	Sorghum	dot blots-genomic DNA	Species	Yao et al. 1990
<i>Phakopsora</i>	Soybean	TaqMan-PCR-ITS/rDNA	Species	Frederick et al. 2002
<i>Pythium</i>	3 species ^b	PCR-ITS/rDNA	Species	Kageyama et al. 1997
<i>Rhynchosporium</i>	Barley	PCR-ITS/rDNA	Species	Lee et al. 2001

^a Seed cultured in liquid medium.

^b Cucumber, sugar beet, and Chinese cabbage.

extraction. Moreover, fungi that do not grow in pure culture may be studied with these techniques. On the other hand, amplifying DNA of nonviable fungi would lead to false-positives for disease potential, but would nevertheless provide useful information for researchers interested in mycotoxins.

4 CONCLUSIONS

Molecular methodologies have been used successfully to address a number of problems in identification of plant pathogenic fungi. Not all of the molecular identification methods have been fully utilized, but this will probably change in the future. Practical application of molecular methodologies is increasing as the instruments become less expensive and the protocols less complex. The demand for rapid, accurate identification of plant pathogens is growing. In addition to the need for such methods in field diagnosis and import/export issues, the threat of plant pathogens as potential bioterrorist weapons has added impetus to the need for rapid identification. The lack of a universally applicable technique that would differentiate all fungi makes rapid identification difficult unless preliminary morphological or physiological information is available. New methods are being developed (Schaad et al. 2002) which hold great promise for rapid identification of many plant pathogens.

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5

The Application of Molecular Markers in the Epidemiology of Plant Pathogenic Fungi

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1 INTRODUCTION

Fungi are present in a variety of forms, in almost every habitat, where they are often specific in their occurrence on particular types of host (or substrate) and ecological niche. Fungi may also become partners with higher plants and enter complex biological relationships with the host (Clay and Kover 1996; Thrall and Burdon 1997). The term pathogen is defined as “a parasite able to cause disease in a host or range of hosts” (Kirk et al. 2001), and pathogenic fungi can occur on all plants. In this chapter this definition of plant pathogenic fungi will be limited to those that cause diseases of living plants, and therefore does not include the fungi involved in the spoilage of stored plant materials that are often referred to as causing post harvest “diseases.” The plant pathogenic fungi consist of a large group of genera and species from diverse areas of the fungal kingdom. Recent developments in the understanding of the evolution of eukaryotic organisms have meant that a number of important plant pathogenic organisms have been reclassified, and are no longer considered as fungi *sensu stricto*. These include the economically important genera of *Phytophthora*, *Pythium*, and other Oomycetes, that are now placed in the Straminipila (Dick 2001). Plant pathogenic fungi have a significant influence on crop productivity. Devastating fungal diseases such as corn smut, potato blight, and black stem rust of wheat can destroy many economically important crops. This situation becomes more critical when the interaction between pathogen and the host

plant involves relatively long periods of intimate interaction without apparent damage to the host, and where the pathogen can persist in asymptomatic hosts for many years (Stanosz et al. 1997). The effects of fungi on living plants vary considerably. At one extreme, damage may be limited to small lesions on leaves or stems [e.g., caused by some *Alternaria* species, see Ellis (1968)], while at the other extreme the plant may be rapidly killed [e.g., by some *Verticillium* species, see Pegg (1984)].

Much work has been done to control fungal disease through selection and breeding programs, the genetic modification of both host and pathogen, and the introduction of resistant varieties [see Stukely and Crane (1994)]. The success of these efforts depends largely on the understanding of genetic variability in the fungal population and monitoring this variation in nature. Many aspects of the biology of the fungi have important consequences at the population level. This particularly applies to the mode of reproduction (i.e., the relative contributions of sexual and asexual, outcrossing and selfing mechanisms), and to hyphal anastomosis between genetically different individuals (Brasier 1991; Glass and Kuldau 1992; Milgroom et al. 1996). A further consideration is that some fungi are predominantly haploid in their vegetative phase, some are diploid, and some are dikaryotic. In the case of pathogenic fungi, genetic variability can be introduced through a variety of mechanisms, either during sexual reproduction or independently of it (Kistler and Miao 1992). Such variability is significant as it can influence

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the host-pathogen interaction as genetic flexibility allows the fungi to readily adapt to changing environmental conditions, including the introduction of new host genotypes.

Understanding the epidemiology of plant pathogenic fungi depends upon on the ability to unambiguously identify sexually produced individuals and asexually produced clones. Classical identification traits, such as morphological, physiological, and disease characters, lack the required sensitivity and accuracy needed for identifying individuals within a population, and this has prevented detailed population studies for many years. Recent developments in molecular techniques now allow population studies on plant pathogenic fungi, and these can be performed with great sensitivity and accuracy. An almost unlimited number of polymorphic loci can be used to detect individual genotypes for the direct assessment of genetic variation in a given fungal population. Application of molecular markers has also allowed the investigation of evolutionary processes in a large number of agriculturally important fungi (Mitchell and Brasier 1994; Milgroom et al. 1996; Valent and Chumley 1991; Vilgalys and Cubeta 1994), and the number and scope of these studies is rapidly expanding.

2 REASONS FOR DETERMINING EPIDEMIOLOGY

As is the case for human and animal diseases, a knowledge of the epidemiology of plant disease can provide important information for its treatment and control, and can lead to the development of forecasting systems [see Shaw 2001; Zadoks and Schein 1979]. In the case of fungal plant diseases, particularly those affecting agricultural crops, the two main areas that need to be determined are the mechanism of spread of the disease, and the specificity and host range of the infecting organism. Fungi are transmitted to, and between, plants by a number of different mechanisms. Many fungi are spread through the soil, some growing from previously infected debris in or above the soil. Others can be transmitted as spores and other propagules through water droplets, or directly as airborne particles. Some plant pathogens may exist on secondary hosts, such as *Fusarium oxysporum* (Armstrong and Armstrong 1958) or on weeds [see Terry and Parker (2001)]. If these hosts are present in or near a crop, they may then act as a reservoir that allows a crop disease to be carried over successive plantings. A few plant pathogenic fungi such as *Ophiostoma* species [see Brasier (1991)] can be spread by insects and other vectors, and some such as *Sclerospora graminicola* (Shetty et al. 1980) remain in the seeds of infected plants, and cause disease in subsequent generations. If the mechanism of transmission is known, this knowledge may be important in the development of control or treatment strategies. Such knowledge is particularly important in the selection of planting material, the preparation and maintenance of planting areas, and the establishment of crop successions and rotation [see Maude (1996)].

In addition to knowledge of the mode of transmission of a plant pathogen, it is also important to be able to determine exactly what is being transmitted in terms of fungal populations. In some cases a single fungal species may consist of a number of different host specific populations. An example of this is the vascular wilt pathogen *F. oxysporum*, where around 170 different forms (referred to as special forms) have been identified. Each of these special forms shows preferential or specific pathogenicity to different hosts, and so *F. oxysporum* special form *cubense* will cause vascular wilt of banana, but would not be expected to cause significant disease on oil palm. Therefore, in order to monitor what fungi are present, and may pose a risk to a crop, it is necessary to know specific details of their pathogenicity. This situation becomes further complicated if there are subpopulations within the pathogen that show differential pathogenicity, either in terms of the degree of damage or the particular cultivars attacked. Such populations are generally described as races, and the ability to differentiate these can make a significant difference to their control. A further factor in considering populations in fungal plant pathogens is whether the population is comprised of meiotic or mitotic forms. Some pathogenic fungi, such as *Fusarium* species, occur almost exclusively in a mitotic (or imperfect) form. In this state, the variability within the population can be assumed to be relatively low, particularly if the disease is the result of a single introduction. Some fungal pathogens, such as *Phytophthora* and the rusts and smuts, are however present on the plant in a meiotic (or perfect) form, and this allows for variability to be introduced into the population at each generation [for example see Duncan et al. (1998)].

3 TYPES OF MOLECULAR MARKERS

Detection of fungi on the basis of visual examination of morphology is highly selective and species-specific identification of fungi and spores is therefore difficult. Molecular techniques present several advantages over the traditional ones and, most importantly, nucleic acid sequences unique to particular organisms can generally be found. As these techniques do not rely on phenotypic examination, gene expression is not required and identification times can be reduced significantly. In the fungi, molecular markers can be derived from both variable and conserved regions of the nuclear and mitochondrial genome, and different markers have been used to define populations at all levels from an individual isolate upwards. Some methods have the potential for the detection of specific genomic DNA sequences directly from initial plant samples, thereby eliminating the requirement to isolate and culture the fungus. Specific molecular markers, probes, and primers have commonly been developed from a variety of DNA sequences including randomly cloned genomic DNA fragments and specific regions such as genes coding for ribosomal RNA (rRNA), virulence factors, and insertion sequences.

3.1 Ribosomal DNA

The nuclear genomes of fungi have a number of particular features. They are relatively small (approximately 13–93 million nucleotide base pairs), and in comparison to higher plants and animals they have a much lower percentage of redundant DNA (about 10–20%) (Lu 1996). Around 30% of the entire fungal genome consists of duplicated regions and genes (Mewes 1997). These repetitive sequences provide potential targets for molecular markers due to their high copy number.

Ribosomal DNA (rDNA), specifically the regions coding for the rRNA subunits and their associated spacers, is one of the most commonly used DNA regions for fungal molecular markers [see Bridge and Arora 1998; Bruns et al. 1991; www.mendel.berkeley.edu/boletus/boletus.html; www.biology.duke.edu/fungi/]. The nuclear-encoded rRNA genes (rDNA) and spacers occur as a gene cluster (typically of 8–12 kb) that is multiply repeated (see Figure 1). The basic unit consists of the genes for the small ribosomal subunit, the 5.8S subunit, and the large subunit. The three genes are separated by two internally transcribed spacers (ITS), and the repeated gene clusters are separated by an intergenic spacer (IGS) that in many, but not all fungi, also contains the gene for the 5S subunit. [see Hillis and Dixon (1991)]. Several restriction sites are conserved in fungal rDNA, and this makes them convenient sequences for cloning [see other reviews by Gargas and DePriest (1996); Hibbett (1992)]. The rRNA cluster has proved to be a good region for deriving molecular markers for many fungi [see Bruns et al. (1991); Hibbett (1992)]. The subunit genes have both conserved and variable domains, and can be used for comparisons of genera, the spacer regions are considerably more variable and can be used for comparisons of species or in some cases specific pathogenic forms (see Table 1). As the cluster is universal and multiply repeatedly, it is a good target for molecular studies. Originally these studies involved obtaining RFLPs with probes hybridized to total genomic DNA digests, and many

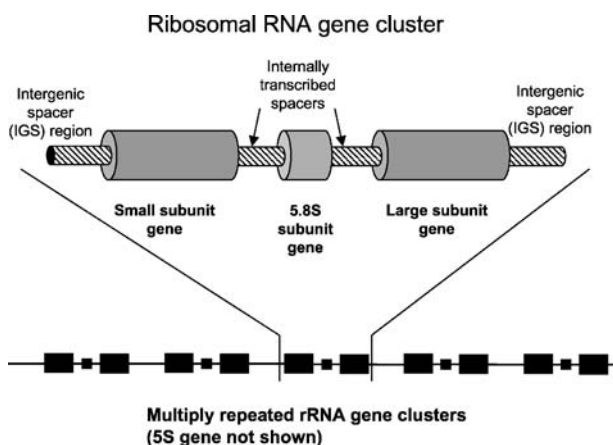


Figure 1 Ribosomal rRNA gene cluster arrangement.

studies of populations and species of plant pathogens were carried out in this way [e.g., Jabaji-Hare et al. (1990), Manicom et al. (1990)]. More recently these studies have largely been replaced by polymerase chain reaction (PCR) based studies, particularly as the rRNA cluster can often be detected in old or contaminated environmental samples [see Bruns et al. (1990)]. The varying levels of specificity of the different DNA regions in the gene cluster also mean that it is possible to amplify fungal DNA directly from samples of infected plant material (Bridge et al. 2000; Gardes and Bruns 1993).

Both the ITS and the IGS regions have been used to develop species-specific primers for plant pathogen detection in plant material [e.g., Brown et al. (1993); Moukhamedov et al. (1993)]. It is becoming increasingly common in rRNA cluster studies to obtain sequences of all the regions of interest. Although knowledge of the complete sequences provides a large amount of information, useful information may be obtained from simple restriction digestions of rRNA amplification products. This approach generally produces relatively simple patterns containing 1–4 bands, and in certain cases these patterns, or individual bands, may be specific for particular pathogens [e.g., Chen (1992)]. It is not possible to list all the work done using ITS and IGS regions to develop molecular markers. One example of this was the study of Mazzola et al. (1996) who developed an oligonucleotide primer set that consistently and selectively amplified a 511 bp fragment in the ITS region that could be used to differentiate between *Rhizoctonia solani* and *R. oryzae*. It should, however, be remembered that RFLP analysis is essentially a one-tailed analysis of variation; and although different patterns indicate that two organisms are different, a common pattern is based on only the position of a few restriction sites. Therefore, identical RFLP band patterns do not imply that the rest of the sequence is identical.

3.2 Protein Coding Genes

There are numerous gene sequences that have been examined in the systematics and phylogeny of plant pathogenic fungi. These include genes for the production of actin, tubulin, elongation factors, cytochromes, proteases, and many others [e.g., Glass and Donaldson (1995); Mehmman et al. (1994); Schoch et al. (2001)]. These genes are generally highly conserved between distant organisms; but can contain short introns that can be very variable in insertion position and number [see Edelman and Staben (1994)]. This variation in introns can be useful as a molecular marker among closely related organisms and this has been investigated for a number of plant associated fungi including *Fusarium*, *Ascochyta*, and *Phoma* (see later).

3.3 Fingerprinting Methods

For the purposes of this chapter DNA fingerprinting methods will be limited to those that have been used with plant

Table 1 Features of commonly used molecular markers

Marker	Taxonomic level resolved	Affected by meiosis ^a
RAPD	Individuals, subspecific groups	Yes
Simple repetitive PCR sequences; micro- and mini-satellite probes and primers	Individuals, subspecific groups	Yes
AFLPs	Individuals, subspecific groups, some closely related species	Yes
mtDNA RFLPs	Subspecific groups, closely related species	No
ITS/IGS region RFLPs	Closely related species, some subspecific groups	Not generally ^b
ITS region sequencing	Some subspecific groups, closely related species	Not generally
rRNA gene sequences	Species, genera, families, phyla	No
Major structural/functional protein genes	Species, genera, families, phyla	No

^aFor many fungi the effects of meiosis on markers have not been specifically considered. Yes and no entries refer to general assumptions.

^bOne reason for the selection of rRNA gene cluster was that it was resistant to crossover. However, there is at least one report of presumed recombination in the rDNA of fungi (see text).

pathogenic fungi to generate largely random PCR fragments from the total genome. These techniques have also been referred to as total genome profiling. One of the oldest and most widely used of such PCR methods is random amplified polymorphic DNA (RAPD) analysis (Welsh and McClelland 1990; Williams et al. 1990). Essentially RAPD analysis relies on the reduction in specificity of the PCR process at reduced temperatures. Total genome DNA extractions are used, and these are amplified with single, short (usually decamer) primers at a reduced annealing temperature. These conditions result in less stringent binding of the primer to the target DNA, and allow the amplification of a number of generally small regions of DNA. These are separated by electrophoresis to give a profile of bands. The RAPD analysis has been criticized as it is not always entirely reproducible, but it has been used extensively for profiling populations of plant pathogens [e.g., Bentley et al. (1995); Cooke et al. (1996)]. In many cases these studies have shown correlations between band patterns and host, disease type or geographical origin, and band patterns have also been used to differentiate between forms of the same fungus causing different disease symptoms [e.g., Pei et al. (1997)]. Another common PCR fingerprinting method that has been used for plant pathogenic fungi is amplification of sequences based on simple repetitive primers. In this method, single short repetitive primers are used at moderate annealing temperatures in order to amplify largely repetitive fragments of the genome. One target site for this method is the flanking region of genes that can contain variable numbers of such repeats. Repeat motifs that have been used for primers have varied from simple 2–3 base repeats such as (CA)₈ and (CAG)₅ (Freeman and Rodriguez 1995; Latge et al. 1998) to more complex sequences including the M13 bacteriophage universal sequencing primer [see Bridge et al. (1997)]. Another method of fingerprinting that has been used with some fungi is based on a group of short repetitive DNA sequences that have been found dispersed throughout the genome of diverse bacterial species [see van Belkum et al. (1998)]. Primers specific to these repetitive sequences produce multiple products in PCR with fungal

genomic DNA, and these can then be separated to provide simple fingerprints. Three particular unrelated families of such repetitive DNA sequences, BOX (54 bp), ERIC (124 bp), and REP, (35–40 bp) have also been used to characterize subspecific populations of different plant pathogenic fungi [see Arora et al. (1996); Toda et al. (1999)].

A relatively recent development in fingerprinting fungi has been the introduction of amplified fragment length polymorphism (AFLP) analysis [see Vos and Kuiper (1997); Vos et al. (1995)]. In this technique, total DNA is digested with restriction enzymes, and then short artificial oligonucleotides (linkers) are ligated to the restriction enzyme sites. Specific primers are then designed that show a particular degree of specificity to the linker sequences, and large fractions of the total DNA can then be amplified as fragments. The AFLP analysis generates many bands, and electrophoresis is usually undertaken in large polyacrylamide gels, it is however, possible to undertake more restricted studies that generate fewer bands and that can be analyzed in smaller electrophoresis systems [e.g., Mueller et al. (1996)]. At the conclusion of RAPD and AFLP analyses PCR bands of interest can be extracted, purified, and sequenced to produce sequence characterized amplified regions (SCARs). The sequence information obtained from SCARs can then be used to develop more specific PCR primers for detection methods [e.g., Dobrowolski and O'Brien (1993); Leclerc-Potvin et al. (1999)].

3.4 Other Total Genome Approaches

Two other methods that have been used to characterize populations of plant pathogenic fungi are the analysis of overall repetitive DNA, and chromosome size and number. When total fungal DNA is digested with frequent cutting restriction enzymes, a large number of fragments of many different sizes are generated, and these appear in a gel as a “smear.” Brighter staining bands can be seen in these smears where there are multiple copies of fragments of the same size.

These bands are, in part, the result of multiple copy DNA such as the rRNA genes and mitochondrial DNA (mtDNA). Differences in the patterns of these bands can be a quick, simple way for differentiating some closely related fungi, and this approach has been used for race designation in *F. oxysporum* sp. f. *pisi* [see Coddington et al. (1987)].

Unlike plants and animals, fungal chromosomes can be very variable, and in many fungal species different isolates may show differences in the number and size of their chromosomes. This has been investigated in some isolates of *Colletotrichum* and special forms of *F. oxysporum*. In *Colletotrichum* variation was found in the number and size of the smaller (type B) elements, and in *F. oxysporum* sp. f. *cubense* variation in chromosome sizes was found between different races and vegetative compatibility groups [see Masel et al. (1993); Ploetz (1990)].

3.5 Mitochondrial Sequences

The mitochondrial (mt) genome has been used extensively in the investigation of population structures in the plant pathogenic fungi. In fungi the mitochondrial genome is a circular structure of between 17 and 121 Kb [see Zimmer et al. (1984); Cuole and Kocher (1999); Grossman and Hudspeth (1985); Lu (1996)]. The fungal mitochondrial genome makes up between 1–20% of the DNA occurring in fungal cells, and generally contains a high proportion of sequences that lack a coding function. In addition it may contain many repeat sequences and introns, and these features can allow for considerable variation in mitochondrial sequences between closely related organisms [see Clark-Walker (1992); Clark-Walker et al. (1987); see chapter numbers 11 and 12 in this book]. As it is present in multiple copies, mtDNA can be a good target for molecular methods. In most cases mitochondrial DNA is inherited unilinearly during meiosis but recombination may occur in some fungi (Wolf 1996). In addition, mtDNA can be transferred independently of the nuclear genome during unstable vegetative fusion (Collins and Saville 1990). The mtDNA can contain GC rich palindromic repeats, but overall, simple GC sequences are relatively rare in fungal mitochondria, and this has been used in differential DNA restriction protocols to generate presumptive mitochondrial RFLPs [e.g., Kouvelis and Typas (1997); Lacourt et al. (1994)]. The mitochondrial genome contains both variable and conserved regions and so sequence information may be used at a variety of taxonomic levels [see Zhou and Stanosz (2001)]. In some cases closely related species may have very different mitochondrial genomes, and one example of this is in the yeast *Saccharomyces*, where mtDNA varies from 24 to 78 Kb between different species [see Grossman and Hudspeth (1985)]. Analysis of mtDNA variation has been used at a variety of different population and systematic levels, and these have correlated with subspecies, vegetative incompatibility groups, different populations and individuals (Gordon and Okamoto 1992; Jacobson and Gordon 1990; Miller et al. 1999).

3.6 Inserted Elements

Both nuclear and mitochondrial genomes in fungi may contain a wide range of inserted elements [e.g., see Edelmann and Staben (1994); chapters 11 and 12]. At the very simplest these may be very short sequences left after transposon insertion and removal, and at the more complex they include a wide range of different transposons [see Daboussi (1997); Daboussi and Langin (1994)]. Inserted elements, particularly introns, have been investigated as potential molecular markers at both the population level and the higher phylogenetic level (DePriest 1993; Neuvéglise et al. 1997).

3.7 Application of Individual Markers

The suitability and resolution of individual markers will depend very much on the fungus being considered, and a marker that is useful for differentiating species in one group of fungi, may be considerably more conserved or variable in other fungal groups. One example of this is the use of presumptive mtDNA RFLPs (AT rich DNA). The mtDNA RFLPs have been found useful for determining species and subspecific populations in some species of *Aspergillus*, whereas in the species *Metarhizium anisopliae* and *Verticillium lecanii* the same approach identifies numerous subspecific groupings (Kouvelis and Typas 1997; Typas et al. 1998; Varga et al. 1994). In the plant pathogen *F. oxysporum* sp. f. *cubense* mtDNA RFLPs have been used to distinguish between different races and supported the theory that the recently determined race 4 was not derived from the existing race 1 or 2 (Thomas et al. 1994). In the basidiomycete *Ganoderma boninense* mtDNA RFLPs have been found useful for defining individuals (Miller et al. 1999), and in some *Phytophthora* species they have been used for determining parental lines (Whittaker et al. 1994).

Differences in the level of variability seen with the same marker from different taxa is not restricted to mtDNA, and appears to be a feature of most markers investigated for population and species level investigations. One example of this is the degree of ITS sequence difference seen between isolates of a single species, or between closely related species. As an example there is generally up to around 5% ITS sequence variation within individual species of *Colletotrichum*, and a maximum of about 23% variation between species (Sreenivasaprasad et al. 1996). In *Rhizoctonia solani*, up to 30% variation in the ITS sequences has been reported between isolates of the same anastomosis group (Kuninaga et al. 1997).

3.8 Combining Markers

In general terms the use of different markers can give rise to a hierarchic system, with particular techniques giving more, or less, resolution than others [see Bruns et al. (1991)], and so it

may be possible to select an appropriate marker for the situation under study. An example of the way in which a broadly hierarchic arrangement of markers can be used for the study of fungal plant pathogens is detailed later with *G. boninense*. This approach will however not always generate consistent results, and one example of this is the group of fungal plant pathogens known as the “*Ascochyta*-complex” that occurs on beans, peas, and other legumes. In this case there are a number of distinct species currently assigned to either the genus *Ascochyta* or *Phoma* (see Table 2). Most of these species can be defined individually from their ITS sequences, and some can in turn be subdivided on the basis of their mtDNA RFLPs. When a single part of the mitochondrial genome is considered there is less variability, and the species can be arranged in three groups. The groupings obtained from RFLPs derived from the β -tubulin gene are less consistent and group some species together, while also showing subspecific groups in others (Fatehi 2000).

3.9 Selection of Molecular Markers

Two of the most important factors that need to be considered in the selection of molecular markers in any study are the taxonomic rank under consideration, and the life cycle of the fungus. As detailed above, a marker that is particularly useful at a certain taxonomic rank for one species may not be useful at the same rank for another species. One explanation for this is that different systematists or plant pathologists have had different species concepts, and so the terms species and subspecies may not be directly comparable between different fungal genera. In some genera, such as *Fusarium*, there has been a tradition of placing different pathogenic forms in the special form category, whereas in others, such as *Colletotrichum* or *Phoma*, there has been a general history of describing new species. Such differences in species concepts may reflect evolutionary ages, or may reflect levels of

variation in other characters. Whatever the reason, there can be significant differences in the degree of variation seen in the molecular markers chosen.

Fungi occur in asexually (imperfect, anamorphic) and sexually (perfect, teleomorphic) reproducing forms, and in some cases both forms are present at the same time (holomorphic). In the imperfect state cell division is solely by mitosis, whereas in the perfect state recombination and meiosis will occur. Recombination and meiosis can have a significant effect on results obtained from some molecular markers (see Table 1). Isoenzyme markers could be expected to be subject to allelic variation under such circumstances, as would many DNA fingerprinting markers. The degree to which a marker will be affected will vary considerably, and one example is the comparison of sibling haploid lines derived from a single dikaryotic fruit body. In these circumstances, the haploid progeny have arisen by meiosis and may show different isoenzyme or DNA fingerprints from the parental material. This has been investigated in the oil palm pathogen *G. boninense* where both RAPD (Pilotti et al. 2000) and simplified AFLP (Figure 2) fingerprints differed both between siblings and between siblings and parent. This variation can then be further compounded through subsequent mating and recombination.

Some molecular markers can be expected to be consistent despite meiosis and recombination. DNA sequences of major structural and functional proteins will be resistant to recombination events, and the rRNA gene cluster is one region generally considered to be maintained under such conditions (Hillis and Dixon 1991). There are however some indications that this is not always the case, and there is at least one report that in some fungi, not only can the rRNA region be affected by crossover, but also that this may occur at a higher frequency than predicted (Selosse et al. 1996).

Nonnuclear markers may be recombination insensitive, and mtDNA has been used to demonstrate a single hereditary line, where the mtDNA was inherited unilinearly (Whittaker et al. 1994). It should be remembered however that this will

Table 2 Features of *Ascochyta* complex species on legumes

Species	mt SSU rRNA size	β -tubulin gene RFLP ^a	ITS sequence ^b	mtDNA RFLPs
<i>P. exigua</i>	749 bp	D	1	Multiple, distinct
	749 bp	A	1	
<i>A. rabiei</i>	660 bp	A	2	Multiple, distinct
<i>A. fabae</i>	660 bp	C	3	Multiple, distinct
<i>A. fabae</i> f. sp. <i>lentis</i>	660 bp	C	4	Single, distinct
<i>A. pisi</i>	660 bp	C	5	Single, distinct
<i>P. medicaginis</i> var. <i>pinodella</i>	645 bp	B	6	Multiple, distinct
<i>A. pinodes</i>	645 bp	B	6	Multiple, distinct
<i>P. subboltschaueri</i>	645 bp	E	7	Single, distinct
	645 bp	F	7	
	645 bp	G	7	

^a Letters A–G designate 7 different RFLP patterns obtained by digestion of a PCR amplified fragment of the β -tubulin gene.

^b Numbers 1–7 designate 7 different RFLP patterns obtained by digestion of the complete ITS1/5.8 s/ITS2 region.

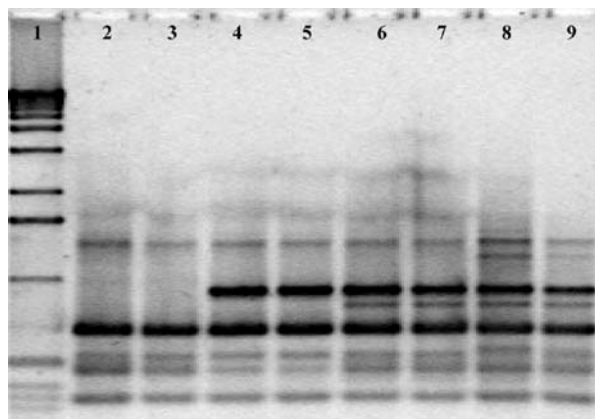


Figure 2 Simple sequence repetitively primed molecular fingerprints for 4 lines of *Ganoderma* derived from a single bracket. Lane 1, molecular size markers; lanes 2 and 3 monokaryotic culture derived from basidiospore a; lanes 4 and 5, monokaryotic culture derived from basidiospore b; lanes 6 and 7, monokaryotic culture derived from basidiospore c; lanes 8 and 9, dikaryotic culture obtained from b and c.

not always be the case, as not all fungi have unilinear mitochondrial inheritance, and in some cases mitochondrial recombination will occur during biparental inheritance (Borst and Grivell 1978). The range and type of variation associated with molecular markers can provide many different tools that can be used for determining the epidemiology of plant pathogenic fungi. At one level, recombination insensitive markers may be available for the detection of a particular taxon in the environment, such as species and pathogen specific probes and primers. At a lower level, recombination sensitive markers may be used to follow individuals or lines, or to determine if a disease is spread by spores or through vegetative growth.

4 ANALYSING DATA

Different molecular markers will generate results in different forms. Simple RFLPs and some fingerprinting methods will produce generally simple band patterns, usually of the order of between one and 20 bands. These patterns can be translated into a simple binary form where each band obtained in the analysis is considered as an independent character, and is scored as present or absent. In most cases, these binary records have then been compared by the use of one or more distance or association calculation, and represented as some form of dendrogram. There is a wide range of coefficients available for such comparisons. These include coefficients that do not consider matching negative characters, and others that provide for a double weighting of common bands to reflect the presence of common restriction sites or primer sequences at each end of the bands [e.g., Nei and Li (1979)].

It should be remembered that some of these coefficients have been described independently on more than one occasion, and others can be related to each other by simple transformations. For example Nei and Li's genetic distance is equivalent to 1-Sorensen's coefficient, and Sorensen's coefficient is mathematically identical to Dice's coefficient [see Bridge and Saddler (1998); Sneath and Sokal (1973)]. Similarly, association coefficients can be related to distance measures, and taxonomic distance can be defined as the square root of 1 minus the simple matching coefficient. It is therefore important if more than one measure is used to ensure that those selected are independent.

Although cluster analysis methods are a common way of showing relationships within and between fungal populations, this methodology does however have some limitations. One obvious limitation with any tree diagram is that all the isolates must be linked, as there is no provision for an isolate that is not related. A second limitation is that cluster analysis is a good technique for showing the membership of a group, it is less precise in showing relationships between groups. This is a particular failing of average linkage based systems, but is also true of most other clustering approaches [see Abbott et al. (1985)]. A further limitation to cluster analysis is the tendency of isolates that are unrelated to the main population to form a separate cluster together, even though they may be only distantly related to each other. Such clusters are sometimes described as sharing only the single property that they are not related to the main population. One way in which some of these limitations can be overcome is by using an ordination-based method such as principle component analysis (PCA). In these methods correlated variance between characters is combined to produce a further set of axes that are essentially made up of additive components of correlated individual characters. Each axis represents a proportion of the total variance in the data, and the placement of isolates is by plotting their positions in relation to the first 2 or 3 axes [see Alderson 1985; Dudzinski 1975]. A refinement of PCA is principle coordinate analysis (PCO). PCO has been shown to be appropriate for binary data, such as obtained from band patterns, and unlike PCA, does not require the use of strictly metric coefficients (Gower 1966; Sneath and Sokal 1973). Unlike a cluster analysis, this does not produce a series of groups, but a scatter-plot where similar isolates may be placed near each other. These ordination methods can behave differently from cluster analysis, and typically are better at representing between group relationships than close within group relationships. One further aspect of PCA is that under some circumstances it may filter random variation from a complex data set, as any correlated variation will tend to be included in the first few axes [see Bridge (1998)].

Band analysis methods are essentially the same for simple and complex patterns. It however becomes necessary to consider band reading software for the very complex patterns that may be produced by techniques such as AFLP, as the large number of bands produced cannot be easily scored by

eye. There are a range of band reading and matching software packages available, and most of these convert band patterns in a gel into densitometric traces where peak presence, height, and shape correspond to band presence, intensity, and thickness, respectively. These packages commonly have manual and automated routines for correcting gels for shift and stretch events, and routines for including standard size markers and other reference bands. Trace data can be readily converted to quantitative values as "x,y co-ordinates" and this is suitable for largely distance based analyses. Quantitative data can also be compared for overall pattern similarity through methods such as correlation coefficients, and concentration independent calculations such as cosine theta [see Feltham and Sneath (1979)].

Analysis of sequence data is more complicated as the likelihood of certain events may also be included in the analysis. The first stage in comparing DNA sequences is to align them to each other. Alignment routines will always seek the best alignment of the sequences being studied, and so the addition or deletion of sequences to a data set will require a new alignment to be made. In determining an alignment, and calculating a measure of difference between the sequences involved it is also necessary to consider the effects of transversions, transitions, and gaps. The bases in DNA strands pair through purine/pyrimidine bonds, and so when aligning sequences, a change from purine to purine or pyrimidine to pyrimidine (transition) may be considered of less importance than a change from purine to pyrimidine (transversion). The importance given to transitions and transversions can therefore be varied to reflect their relative importance, and this may also depend on the particular sequences being considered. When aligning sequences it may be necessary to insert a gap in some sequences to align where insertion/deletion events have occurred. Again, the relative importance of inserting a gap, and also of extending that gap can be adjusted according to the perceived significance of the event in the sequences under consideration [see Thompson et al. (1994)]. It is common with sequence analysis to use phylogenetic techniques to produce trees [see Swofford and Olsen (1990)]. While these approaches are suitable when considering different species and genera, they are less appropriate for comparisons of closely related populations. Analysis of DNA sequence data is an area that is currently receiving further attention, and some of these developments are described in more detail in chapter 33.

5 FOLLOWING PLANT DISEASE: A CASE STUDY

Molecular markers have been used in a wide range of studies with plant pathogenic fungi (see earlier). Although these can be reviewed, the volume of the literature available is considerable, and so a single case study is presented here that illustrates how the molecular epidemiology of a plant pathogen can be related directly to agricultural practice.

One series of studies that has shown the range and limitations of molecular markers in following plant disease epidemiology is the investigation of basal stem rot (BSR) of oil palm by *G. boninense*. BSR was first recognized in West Africa in 1915, and as oil palm was distributed through out the world, it was recorded in many other countries. The first report in SE Asia was in 1931, and since that time BSR incidence has increased to the point where the lack of techniques for management of the disease is considered a major constraint to oil palm production in SE Asia [see Ariffin et al. (2000)]. *Ganoderma* species attack a variety of tropical perennial crops including rubber, tea, and pineapple. In these instances the *Ganoderma* appears to be largely transmitted through the soil, possibly in plant debris, and spreading infection patches may be evident in fields. The species *G. boninense* occurs as a saprophyte on dead palms, particularly coconuts, but appears to be pathogenic only to oil palm. For some years transmission of *G. boninense* in oil palm was believed to be through the soil, as for other species, and disease control was attempted through practices that included digging large pits around infected palms (Turner 1981). The first attempt to use molecular methods to investigate the epidemiology of BSR in oil palm was made in the 1990s, when initial studies were made with iso-enzyme profiles (Miller et al. 1995). Although some enzyme systems initially appeared useful for differentiating species, in *G. boninense* it was found that in general iso-enzyme profiles were either consistent, or showed considerable variation. *Ganoderma* is a basidiomycete that forms polyporoid brackets on the outer surface of infected palms. The brackets are dikaryotic, and basidiospores are produced by meiosis. The mycelial form found in infected tissue is also generally dikaryotic, and some of the iso-enzyme variability may therefore be due to recombination events from the original fusion of monokaryotic basidiospores. However, pectinase isoenzyme analysis identified a characteristic enzyme profile that was consistent for nearly all *Ganoderma* isolates obtained from palm hosts (Miller et al. 1995; 2000). Given the known involvement of pectin and pectin degradation in plant pathology, this finding may indicate a common mode of action for all of the palm associated *Ganoderma* species.

The first DNA based method to be investigated in these studies was analysis of RFLPs derived from presumptive mtDNA (AT rich DNA). This relatively simple technique gave rather unusual results, in that different RFLPs were obtained from different cultures, suggesting considerable heterogeneity in the mitochondrial genome (Miller et al. 1999). The RFLP profiles proved to be consistent among single spore isolates from a single basidiome, and so were considered to provide "parental line" fingerprints, characteristic of the dikaryon. These RFLPs could therefore be used to define sibling families. This assumption was supported by monokaryon and dikaryon intercompatibility studies (Pilotti et al. 2000).

Subsequent investigation of molecular fingerprinting methods including RAPDs and AFLP supported the bulk of the iso-enzyme studies and gave different profiles for isolates

derived from single spores from the same basidiome (Bridge et al. 2000; Pilotti et al. 2000). When these molecular methods were applied to isolates obtained from single plantations and planting blocks it was found that nearly all of the isolates differed from each other, including isolates obtained from adjacent palms. This finding was again supported by intercompatibility studies (Miller et al. 1999; 2000; Pilotti et al. 2000). These results could not have come about as a result of simple mycelial spread in the soil, as vegetative spread could be expected to result in at least some palms being infected by the same isolate. It was therefore concluded that infection could be due to one of two mechanisms, either singly or in combination. The first was that there might have been mycelial spread from multiple inoculum sources, with virtually no cross infection. This would account for the molecular variability recorded, but would also require each infection to be the result of different infected debris. The second possibility was that infections were due to new dikaryons formed from fusion of monokaryons from individual spores [see Sanderson and Pilotti (1997); Sanderson et al. (2000)].

Although the molecular markers studied showed sufficient variability to identify individual isolates for local epidemiology, they did not show sufficient conservation to allow the wider detection of the pathogen in the environment. BSR was considered to be due to the single species *G. boninense*, and although there is considerable uncertainty regarding species concepts in *Ganoderma*, some information is available on sequences within the rRNA gene cluster. Initial studies have shown that the ITS regions are relatively similar across the genus, with most variation being found in the 3' terminal region of the ITS2 sequence (Moncalvo et al. 1995a,b). The ITS sequences obtained from multiple isolates of *G. boninense* showed very little sequence variation, and a short sequence of 16 bases in the ITS2 region was found to be unique to the species. A PCR primer has been derived from this region, and the combination of this and the universal rRNA primer ITS3 allowed the specific amplification of *G. boninense* sequences from cultures, specimens, and infected palm material (Bridge et al. 2001). Current surveys being undertaken by the Oil Palm Research Association in Papua New Guinea with the PCR based diagnostic are detecting *G. boninense* in the internal tissues of recently cut frond bases of young oil palms (Bridge et al. 2000; 2001). This finding suggests that such cut surfaces may provide an entry route for spores. This mode of infection has been reported many times before for fungal pathogens of woody trees, and the initial results from the oil palm research suggest that the developmental state of the oil palm and other factors may also be important in the establishment of infection.

6 CONCLUSIONS

The tools of molecular evolutionary biology and genomics are making it possible to use genetic variation in pathogens and hosts to prevent and treat plant pathogenic fungi. There is a

wide range of molecular features that can be used as markers at the population level for studying plant pathogenic fungi. The different markers will often reflect different levels of variation within and between plant pathogenic taxa, and may also reflect changes resulting from meiosis and recombination. It is therefore possible to study the spread and dynamics of fungal populations on crop plants, and to determine the role of populations of the same fungus occurring on secondary hosts, in the soil or on debris. Molecular markers can also be used to determine the genetic integrity of host or variety specific groups, and can provide information on differences between pathogenic races. This information is fundamental to understanding the spread of fungal plant diseases, and is important in developing disease control strategies. The choice of marker will depend on the particular fungus under study, and the correct choice of markers may also provide information as to the role of spores or particular mating types in epidemiology. Variation in fungal pathogen genotype is the basis for developing methods to identify these pathogens using PCR. Recently, strains/species specific molecular markers/primers have been developed for several plant pathogenic fungi. There is unfortunately no single marker system that can be guaranteed to provide the desired level of discrimination for all fungi, and some initial screening of different methodologies may be required before a full study can be initiated.

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6

Molecular Biology for Control of Mycotoxigenic Fungi

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1 INTRODUCTION

Mycotoxins are fungal metabolites that can contaminate foods and feeds, and exhibit toxic effects in higher organisms (Sharma and Salunkhe 1991) that consume the contaminated commodities. The regulatory guidelines and advisory limits issued by the United States Food and Drug Administration (FDA) on some contaminated commodities can facilitate severe economic losses to the growers. Therefore, mycotoxin contamination of foods and feeds is a serious food safety problem affecting the competitiveness of U.S. agriculture, both domestically and worldwide. Mycotoxins that significantly impact agriculture include aflatoxins produced by *Aspergillus flavus* and *A. parasiticus*, trichothecenes (in particular deoxynivalenol or DON) produced by *Fusarium* spp., ochratoxins produced by *A. ochraceus* and *Penicillium viridicatum*, and fumonisins produced by *F. verticillioides* (synonym, *moniliforme*, as used in some literature cited in the present article) (Brown et al. 1998). Cyclopiazonic acid produced by *A. flavus*, can also be included on this list of significant mycotoxins. Aflatoxins, potent liver toxins, and carcinogens comprise the most widely studied mycotoxins (CAST 1979; Diener et al. 1987; Payne 1998), because of established results in their ability to induce animal diseases, particularly liver cancer in humans [reviewed in Eaton and Groopman (1994)]. However, other mycotoxins such as DON, are of particular concern for the brewing industry which has cutoff levels as low as 0.5 ppm for DON in barley used in malting (Robens 2001). In addition, recognizing the potential for fumonisins to cause animal or human health problems (Marasas 1996), the FDA has now announced the availability of a final guidance document entitled “Guidance

for Industry: Fumonisin Levels in Human Foods and Animal Feeds” in the November 9, 2001, Federal Registry. More than 50 countries have established or proposed regulations for controlling aflatoxins in foods and feeds, and at least 15 countries have regulations for levels of other mycotoxins (Haumann 1995). The FDA has set limits of 20 ppb, total aflatoxins, for interstate commerce of food and feed and 0.05 ppb of aflatoxin M₁ for sale of milk. Because of both food and feed safety concerns and the establishment of regulatory limits on DON and aflatoxins, it is estimated that over \$1.5 billion in crop losses occur annually due to contamination of corn, cottonseed, peanut, and treenuts with aflatoxins and of wheat and barley with DON (Robens 2001).

An association between mycotoxin contamination and inadequate post harvest storage conditions has long been recognized. However, studies have revealed that seeds are contaminated with mycotoxins primarily at the preharvest stage [reviewed in Lisker and Lillehoj (1991)]. Therefore, many current research strategies focus on preharvest control of mycotoxins [reviewed in Brown et al. (1998)]. Maintaining good cultural and management practices that promote the general health of crops can reduce but not eliminate preharvest mycotoxin contamination. For example, insect resistant germplasm, such as corn transformed with the gene encoding *Bacillus thuringiensis* crystal protein (*Bt* maize), reduced levels of fumonisins (Dowd 2000). Irrigation of peanut essentially prevents aflatoxin contamination of this crop, probably by preventing drought stress, known to induce contamination in peanut (Cole et al. 1985). However, optimization of management practices to control mycotoxins is not always possible due to production costs, geographic location, or the nature of the production system for the

particular crop vulnerable to mycotoxins. In addition, even the best management practices are sometimes negated by biotic and abiotic factors that are hard to control and by extremes in environmental conditions. The complex epidemiology of *A. flavus* on corn (Wicklow 1991) can drastically affect the outcome of measures to control aflatoxin contamination on this crop. Therefore, there is an urgent need for development and utilization of strategies involving state-of-the-art technologies to control preharvest mycotoxin contamination. The current article highlights recently published and high-impact research involving molecular-based technologies that has been accomplished and that enhances a host plant resistance strategy for controlling mycotoxin contamination.

2 MYCOTOXIN PREVENTION THROUGH ENHANCEMENT OF HOST RESISTANCE IN CROPS

Preharvest host resistance is a widely explored strategy for combatting fungal attack. By far, most studies aimed at the incorporation of antifungal resistance against mycotoxigenic fungi have been applied toward improvement of resistance against preharvest aflatoxin contamination in corn [reviewed in Brown et al. (1998)]. With corn, the strategy of enhancing host resistance to aflatoxin contamination through breeding has gained prominence because of: (a) the successful identification of germplasm resistant to aflatoxin contamination [reviewed in Brown et al. (1999)] and (b) the significant advances in the identification of natural resistance mechanisms and traits (Brown et al. 1998; 1999; Chen et al. 2001). However, these investigations indicated that resistance to aflatoxin contamination involves multiple chromosome regions and several genes (Davis and Williams 1999). Therefore, attempts to select for resistance traits in the development of commercial corn varieties, while maintaining desirable agronomic characteristics, have been slowed due to a failure to identify expressed genes and proteins involved in resistance. This is especially needed since resistance, thus far identified is in poor genetic backgrounds. Therefore, research is needed to elucidate the biochemical mechanisms that confer resistance in corn kernels and other crops that are vulnerable to aflatoxin contamination. These resistance mechanisms could then be used to enhance germplasm through marker-assisted breeding and/or genetic engineering, two methods for employing the identified traits towards the development of resistant commercially-acceptable crops (Brown et al. 1999). Gaining an understanding of the natural resistance mechanisms in corn could serve as “nature’s lesson” about the specific requirements for seed-based resistance against fungal attack. This information will help efforts to incorporate and enhance resistance in other crops vulnerable to aflatoxin contamination such as cottonseed, peanut, and tree nuts, and will perhaps even help efforts to

enhance resistance against other groups of mycotoxigenic fungi.

2.1 Development of Aflatoxin-Resistance Screening Tools

Several screening tools have been developed and used to facilitate corn breeding for developing germplasm resistant to fungal growth and/or aflatoxin contamination (King and Scott 1982). Inoculation methods employed with corn include the pinbar inoculation technique (for inoculating kernels through husks with *A. flavus* conidia), the silk inoculation technique, and infesting corn ears with insect larvae infected with *A. flavus* conidia. (King and Scott 1982; Tucker et al. 1986). Two resistant inbreds (Mp420 and Mp313E; Scott and Zummo 1988; Windham and Williams 1998) were discovered and tested in field trials at different locations, using the pinbar technique, and released as sources of resistant germplasm.

A rapid laboratory kernel screening assay (KSA) was developed and used to study resistance to aflatoxin production in mature kernels (Brown et al. 1993; 1995). The results of this study indicated the presence of two levels of resistance: at the pericarp and at the subpericarp level. The subpericarp level of resistance was shown to require a viable embryo (Brown et al. 1993). KSA studies further demonstrated a role for pericarp waxes in kernel resistance (Guo et al. 1995; 1996) and highlighted quantitative and qualitative differences in pericarp wax between resistant and susceptible genotypes (Gembeh et al. 2001; Russin et al. 1997). This research was all based on the prior identification, during field studies, of a resistant corn breeding population, GT-MAS:gk (Widstrom et al. 1987).

The KSA also confirmed sources of resistance among 31 inbreds tested in Illinois field trials (Brown et al. 1995; Campbell and White 1995), thus demonstrating that the KSA can be used, at least initially, to rank corn for its field resistance to aflatoxin contamination. Subsequently, the KSA was used as a preliminary screen for resistance to aflatoxin contamination in kernels of maize inbreds selected for ear rot resistance in West and Central Africa (Brown et al. 2001a). The KSA has advantages over traditional field screening techniques (Brown et al. 1995), mainly because of the rapidity of the assay. However, field trials are irreplaceable for confirmation of resistance.

Recently, the KSA was improved by including a method to quantify fungal biomass using the β -glucuronidase (GUS) or green fluorescent protein (GFP) (Du et al. 1999; Windham and Williams 1998; Windham et al. 1999) reporter gene-containing *A. flavus* tester strains. *A. flavus* tester strains were genetically engineered with a gene construct consisting of the GUS reporter gene linked to an *A. flavus* β -tubulin gene promoter for monitoring fungal growth (Brown et al. 1995; 1997) or with the reporter gene linked to an aflatoxin biosynthetic pathway gene which could also provide a quick and economical way to indirectly measure aflatoxin levels

(Brown-Jenco et al. 1998; Payne 1997). Thus, it is now possible to accurately assess fungal infection levels and to predict the corresponding aflatoxin levels in the same kernels, as a result of fungal infection. This technology might be applied to screening for resistance to mycotoxin contamination by other mycotoxigenic fungi. Recently, an *F. verticillioides* strain, containing a GUS reporter gene was used in the KSA to demonstrate that this fungus is inhibited in aflatoxin-resistant genotypes (Brown et al. 2001b). This indicates that some resistance mechanisms may be generic for ear rotting/mycotoxigenic fungi.

2.2 Identification of Resistance-Associated Proteins (RAPs) and Natural Compounds in Corn That Inhibit *Aspergillus flavus* Growth and Aflatoxin Contamination

Developing resistance to fungal infection in wounded as well as intact kernels would go a long way toward solving the aflatoxin problem (Payne 1992). Studies demonstrating subpericarp (wounded-kernel) resistance in corn kernels have led to research for identification of subpericarp resistance mechanisms. Examinations of kernel proteins of several genotypes revealed differences between genotypes resistant and susceptible to aflatoxin contamination (Guo et al. 1998). Imbibed susceptible kernels, for example, showed decreased aflatoxin levels and contained germination-induced ribosome inactivating protein (RIP) and zeamatin (Guo et al. 1997). Both zeamatin and RIP have been shown to inhibit *A. flavus* growth *in vitro* (Guo et al. 1997). In another study, two kernel proteins were identified from a resistant corn inbred (Tex6) which may contribute to resistance to aflatoxin contamination (Huang et al. 1997). One protein, 28 kDa in size, inhibited *A. flavus* growth, while a second, over 100 kDa in size, primarily inhibited toxin formation. When a commercial corn hybrid was inoculated with aflatoxin and nonaflatoxin-producing strains of *A. flavus* at milk stage, one induced chitinase and one β -1,3-glucanase isoform was detected in maturing infected kernels, while another isoform was detected in maturing uninfected kernels (Ji et al. 2000).

In another investigation, an examination of kernel protein profiles of 13 corn genotypes revealed that a 14 kDa trypsin inhibitor protein (TI) is present at relatively high concentrations in seven resistant corn lines, but at low concentrations or is absent in six susceptible lines (Chen et al. 1998). The mode of action of TI against fungal growth may be partially due to its inhibition of fungal-amylase, limiting *A. flavus* access to simple sugars (Chen et al. 1999b) required not only for fungal growth, but also for toxin production (Woloshuk et al. 1997). The TI also demonstrated antifungal activity against other mycotoxigenic species (Chen et al. 1999a). The identification of these proteins may provide markers for plant breeders, and may facilitate the cloning and introduction of

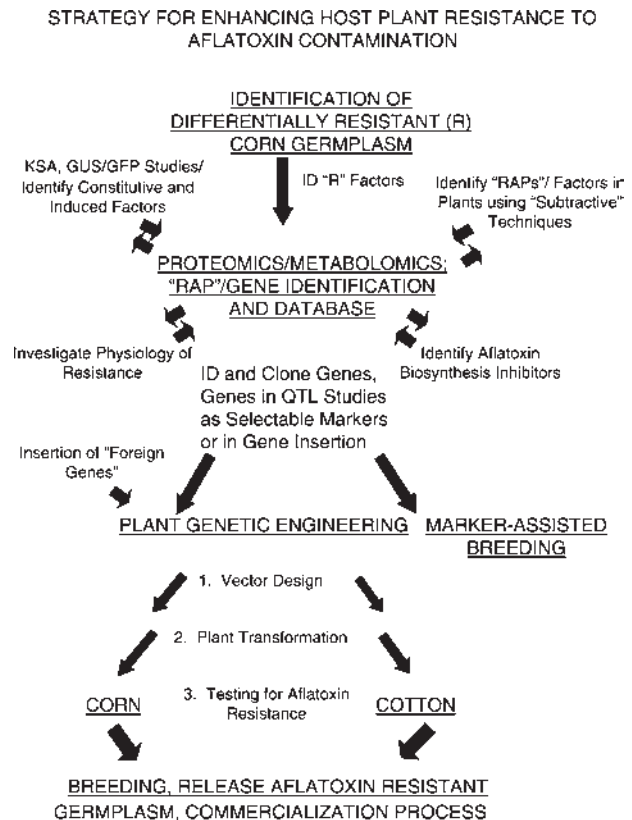


Figure 1 Strategy for enhancing host plant resistance to aflatoxin contamination. The research approach being used to identify and employ resistance factors, such as resistance-associated proteins (RAPs) that are identified in aflatoxin-resistant corn lines. After resistant germplasm is identified, various tools are used to characterize the expression of resistance, such as KSA-based studies, GUS/GFP reporter constructs, and seed physiology studies. These can lead to RAP identification protocols such as proteome analysis. Genes corresponding to RAPs can be cloned, and clones then used for QTL studies, plant transformation, or marker-assisted breeding.

antifungal genes through genetic engineering into other aflatoxin-susceptible crops (Figure 1).

A recent investigation into corn kernel resistance (Chen et al. 2001) determined that both constitutive and induced proteins are required for resistance to aflatoxin production. It also showed that one major difference between resistant and susceptible genotypes is that resistant lines constitutively express higher levels of antifungal proteins compared to susceptible lines. The real function of these high levels of constitutive antifungal proteins may be to delay fungal invasion, and consequent aflatoxin formation, until other antifungal proteins can be synthesized to form an active defense system.

2.2.1 Identification of RAPs Through Proteome Analysis

To increase protein resolution and detection sensitivity by 10–20 fold and, thus, enhance ability to identify more RAPs, a proteomics approach was recently employed. The increased reproducibility, reliability, and accuracy of 2-D gel electrophoresis is due to advances in technology, such as immobilized pH gradient (IPG) gel strips and sophisticated computerized 2-D gel analysis software (Appel et al. 1997; Görg et al. 1998). Endosperm and embryo proteins from several resistant and susceptible genotypes have been compared using large format 2-D gel electrophoresis, and over a dozen such protein spots, either unique or 5-fold upregulated in resistant lines, have been identified, isolated from preparative 2-D gels and analyzed using ESI-MS/MS after in-gel digestion with trypsin (Chen et al. 1999a; 2000; 2002). These proteins can be grouped into three categories based on their peptide sequence homology: (a) storage proteins, such as GLB1, GLB2, and late embryogenesis abundant proteins (LEA3, LEA14); (b) stress-responsive proteins, such as aldose reductase (ALD), a glyoxalase I protein (GLX1), and a 16.9 kDa heat shock protein, and (c) antifungal proteins, which include TI.

Thus far, no investigation has been conducted to determine the possible direct involvement of stress-related proteins in host fungal resistance. Heretofore, most RAPs identified have had antifungal activities. However, increased temperatures and drought, which often occur together, are major factors associated with aflatoxin contamination of maize kernels (Payne 1998). Other studies have found that drought stress imposed during grain filling reduces dry matter accumulation in kernels. This often leads to cracks in the seed and provides an easy entry site to fungi and insects. Possession of unique or of higher levels of hydrophilic storage or stress-related proteins, such as the aforementioned, may put resistant lines in an advantageous position over susceptible genotypes in the ability to synthesize proteins and defend against pathogens under stress conditions. Therefore, the necessary requirements for developing commercially-useful, aflatoxin-resistant maize lines may include, aside from antifungal proteins, a high level of expression of stress-related proteins. Further studies including physiological and biochemical characterization, genetic mapping, plant transformation using RAP genes, and marker-assisted breeding should clarify the roles of stress-related RAPs in kernel resistance.

2.2.2 Natural Compounds That Affect Mycotoxin Biosynthesis

Several compounds have been identified in corn which may have regulatory effects on the aflatoxin and trichothecene biosynthetic process. The compound 4-acetyl-benzoxazolin-2-one (ABOA), which was isolated from maize lines tolerant to *F. graminearum*, strongly inhibited acetyl-deoxynivalinol production at 5 μ M, aflatoxin production at 2 μ g/ml, and feeding by maize weevils at 1000 ppm (Miller et al. 1996).

Steryl esters from maize significantly increased aflatoxin production by some *A. flavus* strains at 0.3 and 1.0 mg/ml (Norton and Dowd 1996). Anthocyanins and related flavonoids, some of which occur naturally in maize kernels, inhibited aflatoxin production by more than 50% at 0.76 mM (Norton 1999). More highly glycosylated forms of the anthocyanins tended to be less effective in inhibiting aflatoxin production (Norton 1999). Carotenoids containing an alpha-ionone type ring tended to be more effective inhibitors of aflatoxin production by *A. flavus*, with some having an I_{50} of about 6 μ M (Norton 1997). Although most strains of *A. flavus* exposed to beta-carotene at 50 μ g/ml had aflatoxin production inhibited by 90% or more, some peanut derived strains were less sensitive (Wicklow et al. 1998). *In vitro* studies indicated plant peroxidase could greatly enhance the ability of plant chemicals to inhibit spore germination and hyphal growth of *F. graminearum* (Dowd et al. 1997). *A. flavus* was considerably more resistant to quinone products potentially produced by plant peroxidases compared to *F. graminearum* and *F. verticillioides* (*moniliforme*) (Dowd et al. 1997). In addition, volatile compounds from corn and cotton, which are products of the lipoxygenase pathway, were shown to have effects upon aflatoxin biosynthesis and fungal development *in vitro* [reviewed in Bhatnagar et al. (2001)].

2.3 Plant Breeding Strategies for Enhancing Host Resistance to Mycotoxigenic Fungi

Several resistant inbreds among the 31 tested in Illinois field trials (Campbell and White 1995) and highlighted through the KSA (Brown et al. 1995), have been incorporated into an aflatoxin-resistance breeding program whose major objective is to improve elite Midwestern corn lines such as B73 and Mo17. In this program, the inheritance of resistance of inbreds in crosses with B73 and/or Mo17 was determined (Hamblin and White 2000; Walker and White 2001; White et al. 1995b; 1998), and in the case of several highly resistant inbreds, genetic dominance was indicated. Overall, results indicated that selection for resistance to *Aspergillus* ear rot and aflatoxin production should be effective, and that development of resistant inbreds for use in breeding commercial hybrids should be successful (White et al. 1995a).

Chromosome regions associated with resistance to *A. flavus* and inhibition of aflatoxin production in corn have been identified through Restriction Fragment Length Polymorphism (RFLP) analysis in three “resistant” lines (R001, LB31, and Tex6) in the Illinois breeding program, after mapping populations were developed using B73 and/or Mo17 elite inbreds as the “susceptible” parents (White et al. 1995b; 1998). In some cases, chromosomal regions were associated with resistance to *Aspergillus* ear rot and not aflatoxin inhibition, and vice versa, whereas other chromosomal regions were found to be associated with both traits. This suggests that these two traits may be at least partially under separate genetic control. Also, it was observed that

variation can exist in the chromosomal regions associated with *Aspergillus* ear rot and aflatoxin inhibition in different mapping populations, suggesting the presence of different genes for resistance in the different identified resistance germplasm. The RFLP technology may provide the basis for employing the strategy of pyramiding different types of resistances into commercially viable germplasm, while avoiding the introduction of undesirable traits. Another Quantitative Trait Loci (QTL) mapping program was undertaken using a mapping population created from a resistant inbred Mp313E and a susceptible one, Va35 (Davis and Williams 1999), and regions on chromosomes, associated with resistance to aflatoxin contamination, were revealed. Other work using this technology is attempting to pyramid insect and fungal resistance genes into commercial germplasm (Guo et al. 2000; Widstrom et al. 2003).

Breeding strategies to enhance resistance to *A. flavus* infection are being carried out in other crops vulnerable to aflatoxin contamination such as peanut and tree nuts. Promising sources of resistant peanut germplasm have been identified from a core collection representing the entire peanut germplasm collection (Holbrook et al. 1995), although resistance screening has proven to be a difficult task with this crop (Holbrook et al. 1997). Promising peanut germplasm has less than acceptable agronomic characteristics, and is thus being hybridized with lines with commercially acceptable features. Resistant lines also are being crossed to pool resistances to aflatoxin production. Thus, some success has been achieved in identifying resistant peanut germplasm, and field studies are being conducted by various researchers to verify this trait.

Among tree nuts, strategies for controlling preharvest aflatoxin formation by breeding for host resistance have been studied mainly in almonds (Gradziel et al. 1995). The approach has been to integrate multiple genetic mechanisms for control of not only *Aspergillus* spp. but also insects. Resistance to fungal colonization has been shown to be present in the undamaged seed coat of several advanced breeding selections and is further being pursued through breeding/genetic engineering of resistance to *A. flavus* growth in kernel tissues. Genotypes are also under development that produce low amounts of aflatoxin following fungal infections (Gradziel and Dandekar 1999).

Naphthoquinones in walnut hulls delayed germination of *A. flavus* conidia and were capable of inhibiting growth of the fungus at higher concentrations (Mahoney et al. 2000; Molyneux et al. 2000). These compounds also appeared to have a regulatory effect on aflatoxin biosynthesis and may be involved in resistance to aflatoxin contamination of walnut. Results seen here could lead to breeding applications to enhance resistance in walnut to aflatoxin contamination using naphthoquinone derivatives as selectable markers.

Investigations have also been conducted with figs and pistachios to identify the mode of infection of the crops by *A. flavus* and develop strategies to identify germplasm with agronomically desirable characteristics and resistance to fungal infection (Doster et al. 1995). However, until more is

known about the nature of selectable resistance markers associated with reduced aflatoxin contamination in crops other than corn, breeding for insect resistance, or better management of insects which vector aflatoxigenic fungi may be a more viable immediate approach to manage aflatoxin contamination.

Recent studies indicate that naturally occurring resistance may reduce invasion of crops by other economically important mycotoxigenic fungi. For example, resistance to head blight in wheat varieties was correlated with a reduction in contamination with DON (Bai et al. 2001). Further investigations utilizing differentially resistant wheat germplasm may lead to the identification of selectable resistance markers useful in breeding for reduced DON contamination in wheat.

2.4 Genetic Engineering Strategies to Enhance Host Resistance to Mycotoxin Contamination

Plant breeding for resistance is practical when a large germplasm pool exists with differential resistance in the crop, such as exists in corn. However, genetic engineering for resistance may be essential for crops such as cotton which seems to have little resistance to aflatoxin contamination of its seed (Cotty 1989). Extensive research has focused upon identifying genes encoding antifungal proteins effective against mycotoxigenic fungi. Bacterial chloroperoxidase (CPO) (Wolffram et al. 1988) and its gene have been evaluated both *in vitro* in laboratory assays (Jacks et al. 1999) and *in vivo* in enhancing fungal disease resistance in transgenic tobacco plants (Rajasekaran et al. 2000b). In *in vitro* bioassays using *A. flavus* as the test organism, CPO greatly reduced the viability of *A. flavus* conidia (Jacks et al. 1999) and transgenic tobacco expressing the CPO gene demonstrated significant resistance to attack by *Colletotrichum destructivum* (Rajasekaran et al. 2000b).

In another study, a small lytic peptide, D4E1, demonstrated broad spectrum antimicrobial activity and convincing inhibitory activity against *A. flavus* *in vitro* (Rajasekaran et al. 2001), thus indicating the possibility of transforming plants with the gene encoding D4E1 to reduce infection of seed with toxigenic fungi. In further substantiation of this strategy, the D4E1 gene when transformed into tobacco was shown to greatly enhance resistance to *C. destructivum* (Cary et al. 2000). Cotton is being transformed with CPO and D4E genes with the hope that aflatoxin contamination of cottonseed can be reduced (Chlan et al. 1999; Rajasekaran et al. 1999; 2000a).

Mechanisms of mycotoxin biosynthesis and regulation have been investigated extensively (Bhatnagar et al. 2002; Cleveland and Bhatnagar 1992; Desjardins and Proctor 2001). The goal is to identify weak links that can be exploited to control mycotoxin contamination through genetic engineering of plants. The finding that trichothecenes contribute to the virulence of *F. graminearum* on wheat and maize has

identified such a weak link. If production of trichothecenes increases pathogen virulence, then increased plant resistance to the toxin should increase plant resistance to the pathogen. Three genes that increase plant resistance to trichothecenes have recently been identified, and whether such genes also can increase plant resistance to *F. graminearum* is under investigation. Two trichothecene resistance genes are fungal genes that encode proteins that reduce the toxicity of trichothecenes. *TRI101* from *F. sporotrichioides* encodes trichothecene 3-O-acetyltransferase which converts trichothecenes to less toxic derivatives (Kimura et al. 1998; McCormick et al. 1999). *PDR5* from yeast encodes a multidrug resistance transporter protein that transports trichothecenes extracellularly and is similar to *TRI12*, a trichothecene biosynthetic gene (Alexander et al. 1999; Balzi et al. 1994). Transgenic expression of either *TRI101* or *PDR5* increased resistance of tobacco to trichothecenes (Muhitch et al. 2000). Wheat and barley lines expressing *TRI101* and *PDR5* are being tested for resistance to *F. graminearum* (Okubara et al. 2000).

Trichothecenes are potent inhibitors of protein synthesis and are believed to bind to the 60S ribosomal protein L3 (RPL3). A rice gene encoding RPL3 was modified to change amino acid 258 from tryptophan to cysteine, a change that confers trichothecene resistance to yeast. Transgenic expression of the modified *Rpl3*, increased resistance of tobacco to trichothecenes (Harris and Gleddie 2001). Maize, wheat, and barley lines expressing the modified *Rpl3* gene are being tested for resistance to *F. graminearum* (Harris and Gleddie 2001).

No analogous weak link in the aflatoxin biosynthetic pathway has been discovered that can be exploited in a similar host resistance strategy, nor has a clear role for aflatoxin in fungal virulence been demonstrated. However, the aflatoxin biosynthetic pathway and the gene cluster comprising genes that govern this pathway, including a key regulatory gene (*aflR*), have been characterized (Bhatnagar et al. 2002). Also, the regulation of these genes during invasion of the host plant is being investigated using a genomics approach. This approach is based upon the fact that certain plant-derived natural products apparently have regulatory effects on aflatoxin biosynthesis [as recently reviewed in Bhatnagar et al. (2001) and reported in recent publications cited in this article: Miller et al. 1996; Norton 1999; Norton and Dowd 1996; Wicklow et al. 1998].

Genetic engineering may provide innovative solutions to prevent the accumulation of fumonisins in *Fusarium*-infected maize. One approach currently under development is detoxification of fumonisins by enzymes introduced into maize via genetic engineering. Enzymes that detoxify and degrade fumonisins have been identified from *Exophiala spinifera*, a black yeast found on moldy maize kernels. The initial steps in fumonisin detoxification are ester hydrolysis followed by oxidative deamination to produce derivatives that lack the free amino function that is believed to be important for toxicity (Blackwell et al. 1999). Genes encoding the deesterification and deamination enzymes have been cloned

and are being expressed in transgenic maize to evaluate their effect on fumonisin accumulation and ear rot symptoms (Duvick 2001).

The gene encoding the antifungal protein, TI, previously shown to be correlated with corn kernel resistance, was transformed into and expressed in both tobacco and cotton. Fungal growth inhibition assays of transgenic tobacco expressing TI protein showed efficacy against *A. flavus*, but not at the levels observed with extracts from tobacco transformed with genes encoding CPO or D4E1 (Cary et al. 2000; Rajasekaran et al. 2000b). The gene encoding TI also has been transformed into cotton, but no inhibitory activity has yet been noted in extracts from transgenic plants.

It is well documented that insect injury can provide a port of entry by mycotoxigenic fungi and that crops containing the *B. thuringiensis* (*Bt*) gene encoding an insecticidal protein, have shown reduced levels of mycotoxin contamination (Dowd 2000). Currently, a binary vector is being used in this laboratory to express both the antifungal D4E1 gene and a synthetic anti-insecticidal gene, *cryIA(c)*, of *B. thuringiensis* in tobacco and cotton. Successful expression of these genes under independent promoters should provide both fungal and insect resistance in cotton, thus potentially reducing the amount of fungal entry through insect injury sites as well as retarding the growth of the aflatoxin producing fungus in cotton bolls and seed.

3 CONCLUSIONS

Since it is unlikely that preharvest mycotoxin contamination of crops will be reduced significantly through careful cultural practices, control of these problems will likely be dependent upon the development and introduction into the commercial market, of germplasm, resistant to the growth of mycotoxigenic species, and/or biosynthesis of toxins by these species. The identification of resistance traits in corn and other crops can, through marker-assisted breeding, facilitate a more rapid development of resistant, commercially-acceptable germplasm. Genetic engineering provides a tool especially useful for introducing resistance genes into crops with little natural genetic diversity (e.g., cotton), and for testing the efficacy of putative resistance genes.

Studies identifying compounds that affect mycotoxin biosynthesis offer hope to researchers. Limiting fungal growth in crops is an important aspect of host resistance, however, obtaining zero growth of fungi capable of exploiting a variety of different substrates, such as the facultative pathogen *A. flavus*, may be difficult to achieve. Therefore, the identification of a natural compound that blocks mycotoxin biosynthesis might be the closest we come to discovering a "magic bullet." Nevertheless, the investigations discussed in this chapter, using molecular-based technologies to identify and characterize various resistance mechanisms in crops susceptible to mycotoxin contamination, and against different mycotoxigenic fungi, are building a foundation which can lead to the implementation of a successful gene pyramiding

strategy to produce mycotoxin-resistant, commercially-attractive crops.

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Biotechnological Potential of Entomopathogenic Fungi

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1 INTRODUCTION

Fungi have been known to attack insects and mites for thousands of years. Although the causative agent of fungal disease of insects was not always understood, insects infected with fungi were recorded by the Chinese in the seventh century (Tanada and Kaya 1993) and drawings of *Cordyceps* infections abound in early 18th and 19th century literature. The first experimental demonstration of a microbe as a disease-causing organism was by Agostino Bassi, published in 1835–1836, in Italy, with the silkworm pathogenic fungus, *Beauveria bassiana*. He demonstrated that the fungus causes insect death and could be transmitted to other silkworms. It was not long after the first demonstration of the devastating impact of an entomopathogenic fungus on a beneficial insect that it occurred to researchers that disease may be a useful method for control of insect pests. Pasteur is credited with the early proposition that fungi could be used to control a pest insect. He proposed that a fungus could be used against *Phylloxera* in grapevines, a pest eventually controlled using copper solutions. However, it was the Nobel Prize winning researcher, Elie Metchnikoff, who first developed a fungus as a practical control agent for application to a pest. Working in Russia from 1878, Metchnikoff developed the fungus *Metarhizium anisopliae* for control of the cereal cockchafer, *Anisoplia austriaca*, then a devastating pest. Metchnikoff carried out the first successful infection experiments with larvae of *A. austriaca* and the sugar beet weevil, *Cleonus punctiventris*, and initiated mass production of the fungus for field experiments (Zimmermann et al. 1995). With the mass production of *M. anisopliae*, Metchnikoff applied a biotechnological approach to entomopathogenic fungi for the first time, a precursor to the development of biopesticides. The first actual field application of *M. anisopliae* in Russia was left to Krassilstchik (1888) who mass produced the

fungus and applied it in the field for sugar-beet weevil control.

Despite the early discovery of their potential, it was only recently that entomopathogenic fungi have been utilized successfully in biocontrol programs. The fungi are often effective as natural control agents, but their activity is very dependent upon environmental conditions. Many have restrictive temperature ranges for germination, infection, and sporulation, or high humidity requirements for sporulation and spore germination. In some cases, the infective stage is not robust and, as many of the most promising candidate fungi for pest control have lost the ability to form persistent stages such as resting spores, storage and application can be problematic. Variation within species or clusters of species has not been well understood, thus strain selection has not often been attempted or not been possible. The application of biotechnology to the study and development of entomopathogenic fungi has the potential to overcome some of these limitations. Biotechnology has contributed to all areas in the development of entomopathogenic fungi as biocontrol agents, from identification to formulation. This chapter reviews the contribution of biotechnology to the development of entomopathogenic fungi.

2 BIOPESTICIDE POTENTIAL OF ENTOMOPATHOGENIC FUNGI

There are a number of methods for using entomopathogenic fungi against insect pests. Eilenberg et al. (2001) recognized four main strategies: (a) classical biological control, the intentional introduction of an exotic strain for long term, unmanaged control, (b) inoculative biocontrol, the intentional release of endemic strains for long-term unmanaged biocontrol of endemic pests, (c) inundative biological control,

Table 1 Biopesticides based on entomopathogenic fungi (Milner 2000; Reddy et al. 2001; Shah and Goettel 1999)

Species and product name	Targets	Company	Main countries and reference
<i>B. bassiana</i>			
AGO Biocontrol	Coleoptera, Homoptera, Lepidoptera, Diptera	Ago Biocontrol	Columbia
Bassiana			
Biorin	Lepidopteran caterpillars	Biotech International	India www.biotech-int.com
BotaniGard and	Homoptera/Heteroptera, thrips, Coleoptera,	Emerald BioAgriculture (ex. Mycotech Corporation)	USA
Mycotrol	Lepidoptera and Orthoptera	Eric Schweizer Seeds Ltd.	Switzerland
<i>Beauveria</i> Schweizer	Turf/grassland, fruit growing, viticulture and horticulture		
Dispel	Podborers		India
Ostrinil	Corn earworm <i>O. nubilalis</i>	Natural plant protection	France
<i>B. brongniartii</i>			
AGO Biocontrol	Coleoptera, Homoptera, Lepidoptera, Diptera	Ago Biocontrol	Columbia
Beauveria 50			
Engelingspilz	<i>Melolontha melolontha</i>	Andermatt Biocontrol, AG	Switzerland
<i>M. anisopliae</i>			
Ago Biocontrol	Lepidoptera, Coleoptera, Homoptera, Orthoptera	Ago Biocontrol	Colombia
Metarhizium 50			
BIO 1020	Vine weevil	Bayer AG/	Germany, USA
BioGreen	<i>A. couloni</i> (red-headed cockchafer)	BioCare Technology Pty. Ltd.	Australia
BioCane	Greyback canegrubs	BioCare Pty. Ltd.	Australia
Green guard	Locust and grasshopper	Seed Grain and Biotechnology	Australia
		Australia Pty. Ltd.	
Green muscle	Locusts and grasshoppers	Biological Control Products SA PTY	South Africa
<i>Metarhizium</i>	Turf/grassland, fruit growing, viticulture and horticulture	Eric Schweizer Seeds Ltd.	Switzerland
Schweizer			
Taenure	Root weevils, grubs, ticks, immature thrips, white flies	Earth BioSciences Inc.	http://www.taensa.com/products-taenure.html
<i>L. giganteum</i>			
Laginex	All mosquito larvae	AgraQuest, Inc.	Colombia, USA
<i>Nomuraea rileyi</i>			
AGO Biocontrol	Lepidoptera	Ago Biocontrol	Colombia
<i>Nomuraea</i> 50			
<i>P. fumosoroseus</i>			
AGO biocontrol	Coleoptera, nematodes	Ago Biocontrol	Colombia
<i>Paecilomyces</i>			
PFR-97 biological insecticide	Whiteflies, aphids, thrips, spidermites	Thermo Trilogy Corporation	European Union, USA
<i>V. lecanii</i>			
Ago Biocontrol	Homoptera, Diptera	Ago Biocontrol	Colombia, USA
Verticillium 50			
Mycotal	Whiteflies, some activity against thrips	Koppert Biological Systems B.V.	Netherlands, UK, Switzerland, Finland, Norway, Denmark
Vertalec	Aphids	Koppert Biological Systems B.V.	Netherlands, UK, Switzerland, Finland, Norway, Denmark

the use of fungi to limit pests when control is achieved exclusively by the mass release of the organism, and (d) conservation biological control, modification of the environment to enhance fungal infection. Inundative biological control usually relies on the development of biopesticides based on pathogenic microbes, which is the most obvious application of biotechnology to entomopathogenic fungi.

2.1 Biopesticides Based on Fungi

Application of fungi in mass inoculations against insect pests began with Krassilstchik in 1888. Indeed, such was the optimism at that time Krassilstchik confidently predicted “the idea of controlling insects by means of artificially induced epidemics, an idea expressed some 20 years ago by scholars, has become a practically feasible one, which in the future will be perfected and broadly utilized” (Krassilstchik 1888). Unfortunately, progress has been much slower than what was predicted. While progress in the early 1900s was promising, the discovery and application of effective chemical pesticides in the 1930–1940s reduced interest in the use of insect pathogens. Insect pathogenic fungi were more difficult to use, and it was not until environmental and health problems associated with the use of chemical insecticides became apparent in the 1960s that interest in fungal biopesticides again increased. Currently, most biopesticides based on entomopathogenic fungi in the market include either *B. bassiana* or *M. anisopliae* (Table 1). These two species, the so-called muscardine fungi, have broad host ranges, although individual strains may be restricted in the number of insect species that they can attack. These species are relatively easy to produce, as they produce vast amounts of asexual conidia in culture as well as on insects. They are generally considered to have low mammalian toxicity and few nontarget impacts have been reported (see Section 6).

A number of biopesticides have been based on the white muscardine fungus, *B. bassiana* (Table 1). The better known products are those of Emerald BioAgriculture (a merger between Mycotech Corporation and Auxin Corporation) such as BotaniGard® and Mycotrol®. BotaniGard is a liquid emulsion formulation of *B. bassiana* conidia while Mycotrol is based on powdered conidia. There are a number of other products based on *Beauveria* spp. registered around the world. In France, Ostrinil™, based on *B. bassiana* has been produced for many years for corn earworm (*Ostia nubalis*) control, while in India the biopesticide Dispel is sold for control of podborers (Reddy et al. 2001). Similarly, a number of biopesticides are based on the green muscardine fungus, *Metarhizium* spp. Biopesticides based on *Metarhizium* spp. have had a long (if not always successful) history. In the 1980s, Bayer Corporation produced a biopesticide, Bio1020, which was a formulation of *M. anisopliae* with excellent shelf life and application potential. It was primarily developed for control of black vine weevil (Reinecke et al. 1990), but was tested against a number of other pests [e.g., Tabata (1992)]. However, the product was not commercially successful for a

number of reasons (Reinecke et al. 1991) and was unavailable for many years. Recently, Bio1020 has reappeared in the market as Taenure™, sold by Earth BioSciences (<http://www.taensa.com/products-taenure.html>).

A recent success story for biopesticides has been the development of novel strains of *M. anisopliae* var. *acridium* for locust control in several countries. Initially, a strain of this fungus was developed in Africa under a program called LUBILOS, which led to the biopesticide “Green Muscle” (Lomer et al. 2001). This program has inspired development of indigenous strains of *M. anisopliae* var. *acridium* in other countries. For example, in Australia the success of the LUBILOS program has been duplicated with the development of Green Guard™ based on an Australian isolate of *M. anisopliae* var. *acridium* (Milner 2000).

Other biopesticides based on *Metarhizium* are sold around the world. In Australia, an isolate of *M. anisopliae* has also been developed as a commercially available biopesticide for the control of sugarcane scarabs, particularly the grayback canegrub, *Dermolepida albobirtum*. BioCane™ is effective when applied at 33 kg/ha (1×10^{10} conidia/m) before filling-in of the planting furrow (Samson et al. 1999), giving 50–60% control of grayback larvae (Logan et al. 2000). In Columbia, a product based on several entomopathogenic fungi (Micobiol) has been tested against *Prodioplosis longifila* (Diptera: Cecidomyiidae) infesting tomatoes, but was not as effective as conventional control products (Delgado et al. 1999).

Several other entomopathogenic fungi have been developed as commercially available biopesticides (Table 1). Fungi such as *Paecilomyces* and *Verticillium* are similar in action to *Metarhizium* and *Beauveria*. However, a more unusual fungus for development as a biopesticide is the aquatic active, *Lagenidium giganteum*. This Oomycete fungus is active against mosquito larvae and has been developed into the biopesticide Lagenex™ in California and is now sold by AgraQuest, Inc. In field trials against the mosquito *Culex quinquefasciatus*, Lagenex™ compared favorably with Vectobac™ (based on *Bacillus thuringiensis israelensis*), in terms of persistence of control (Hallmon et al. 2000). No biopesticides are currently produced using any species of the Entomophthorales, which is a large order containing mainly entomopathogenic fungi. These fungi, which typically forcibly discharge their primary conidia, often cause large-scale epizootics among insects. This suggests huge potential for development of this group of fungi as mass applied biopesticides. However, problems in production and stabilization of the fragile conidia or the more durable resting spores have not been overcome, and economic products are not feasible at this time.

Biopesticide production has increased in many Central and South American nations and some are not strictly commercial. For example, in Cuba where, as a result of the trade embargo, it has been difficult to obtain cheap chemical pesticides, a biopesticide production industry has grown to fill the gap. Under the Cuban Ministry of Agriculture, decentralized laboratories provide insects, nematodes, and

entomopathogens (bacteria, fungi, and viruses) throughout Cuba's 15 provinces (Rosset and Moore 1997). These "Centres for the Production of Entomophages and Entomopathogens" (CREEs) have facilitated the rapid adoption of IPM systems in crops previously managed under pesticide-based systems. Several fungal entomopathogens are produced for a number of pests, including *B. bassiana* for control of coleopteran pests and *Verticillium lecanii* for whitefly, *Bemisia tabaci*. In 1994, 781 metric tonnes of *B. bassiana*, 196 of *V. lecanii* and 142 of *M. anisopliae* were produced by the production centers. Similarly, biopesticides based *M. anisopliae* are produced by some Central American sugar plantations for the control of pests. The sugar companies have their own production facilities for *Metarhizium* and *Beauveria* [e.g., Badilla (2000) and Grimm (2001)]. These localized production facilities produce sufficient quantities of fungal inoculum for control of pests such as the coffee berry borer, the diamondback moth, and spittlebugs.

2.2 Production, Formulation, and Application

Production of entomopathogenic fungi has not advanced greatly beyond the use of simple grains as substrates for the Deuteromycete fungi, such as *Metarhizium* and *Beauveria*. For many other entomopathogenic fungi, especially among the Entomophthorales, growth in culture is difficult or has yet to be achieved. Both liquid and solid substrates have been substantially investigated (Burgess 1998). Two-stage systems, where both liquid and solid substrates are used, have occasionally proved successful. For example, fermentation to produce hyphae to use as starter cultures is now a widespread practice. There are number of advantages to using liquid cultures as starter cultures: (a) the competitive ability of the fungus is enhanced, reducing the risk of contamination from other microbes, (b) growth is more rapid in the early stages, (c) the liquid culture can be screened for contamination prior to use, and (d) the liquid ensures even coverage of the solid substrate (Jenkins et al. 1998). Liquid starter cultures are commonly used to begin solid substrate production. However, experience with *M. anisopliae* in our laboratory is probably typical of many other laboratories, where inoculation of rice grains with fermenter broth of *M. anisopliae* hyphal bodies gave no improvement in production over the use of conidia from plate cultures (Glare et al. unpublished data). Production on grains is generally in the range of 10^8 – 10^{10} conidia/g of dry substrate [e.g., Feng et al. (1994)], taking between 2 and 3 weeks to reach maturity at optimal temperatures. Interestingly, *Metarhizium* and *Beauveria* sporulate better when the substrate is relatively poor in nutrient content. When grains were supplemented with sugars and yeast additives, less conidia per gram of substrate was obtained than with grains alone (Nelson et al. 1996). Similarly, in Brazil, *M. anisopliae* has been found to produce conidial yields of 5–15 times higher using rice bran/rice husk substrate mixtures than yields

usually obtained for rice grains, with viabilities of higher than 85% (Dorta et al. 1990).

The production of Green Muscle™ *M. anisopliae* for locusts in Africa used a two-stage production system with fermenter production of inoculum used to inoculate rice (Cherry et al. 1999). The process requires relatively low capital investment, but has high labor costs. As with production of most fungi, high variability in yield was reported between batches, and this variability was only partly accounted for by temperature and duration of incubation (Cherry et al. 1999).

A method that showed some promise in the 1980s was the preparation of dried mycelium. Hyphal bodies were harvested by filtration, washed with water to remove culture medium residue, and then coated with a sugar solution before drying. This method was used with *M. anisopliae* and *B. bassiana* (Pereira and Roberts 1990). They found that conidial production was similar to other methods after storage for up to 4.5 months at 4°C and could be superior to other methods with respect to storage at room temperature, however no products at present use this technology.

The Emerald Bio production plant (previously Mycotech) in Butte, Montana, represents the technological end of the production of entomopathogenic fungi. Largely utilized for the production of *B. bassiana*, it is a "state of the art" dedicated facility, with in-line sterilization and large temperature controlled growth facilities. The actual production method is a trade secret, but is based on fermented starter cultures and solid substrate growth and sporulation. This highly technical facility contrasts with the numerous low technology "factories" producing fungi for insect control in China and much of Latin America.

Compatibility between production, formulation, and application techniques is vital for the successful use of microbial biopesticides. The LUBILOSA program for locust control used *Metarhizium* in oil formulations and ULV spraying, which required lipophilic conidia for easy suspension in oils (Jenkins et al. 1998). While production of submerged conidia was seen as having many advantages, the resulting conidia were hydrophilic and lost viability quickly. Therefore, production on grains remains the standard with the locust products. For many years, approaches to the use of entomopathogenic fungi involved point release ("classical biological control") or simple application of conidia, formulated in water with wetting agents. However, appropriate formulation can advance entomopathogenic fungi from curiosity to effective biocontrol agents. It has been an area that has benefited from the application of biotechnology. Formulation has been important in terms of improved survival during storage, persistence in the field (such as UV and desiccation tolerance), and ease of application.

The LUBILOSA program, where *M. anisopliae* var. *acridium* was developed into a biopesticide for locust control in Africa, is an excellent example of formulation overcoming environmental constraints. As locusts live in hot, dry climates and *M. anisopliae* conidia require high humidity to germinate, it seems impossible that an entomopathogenic fungus could

successfully control the pest. However, formulating *Metarhizium* conidia in nonevaporative diluents such as oils allowed the conidia to attach and germinate on susceptible locusts. *M. anisopliae* oil formulations are especially useful at low relative humidities (Bateman 1997). There have been several interesting studies on formulating hyphal material from members of the Entomophthorales. These fungi, because of the fragile nature of the mycelium and conidia, pose a much greater formulation problem than most of the Deuteromycetes, which has contributed to their lack of commercial success. McCabe and Soper (1985) patented a process of drying the mycelium of *Zoophthora radicans* and coating it with sugar, as a method for long-term storage. More recently, Shah et al. (1998) demonstrated algination as a method for formulating *Erynia neoaphidis* mycelium. An important area of formulation and production is the drying of conidia of entomopathogenic fungi. Moore et al. (1996) have shown that survival of conidia of *M. anisopliae* was highest at low (<5%) relative humidity, therefore, this is an important aspect of producing a stable product.

Use of appropriate application techniques that are suited for the application of biopesticide to the target pest is an obvious, but often neglected aspect of biopesticide use. Advances in chemical pesticide applications have slowly filtered through to use with biopesticides, such as ultra-low volume (ULV) application of *M. anisopliae* for locust control (Lomer et al. 2001). Nonevaporative diluents such as oil are required to take advantage of ULV spraying. Rotary atomizers have been used for low volume oil formulations and ULVs for less than 5 l/ha. Electrostatically-charged ULV sprayers have been investigated for better coverage on leaf undersides (Sopp et al. 1989). Generally, application of fungal-based biopesticides has been with conventional equipment and research has focused on spray coverage, droplet size, and placement (i.e., penetration to the underside of leaves). Hydraulic spray systems have been used to apply water-based formulations on crops, air-blast and air-assist technologies are primarily used for low volume applications in fields and orchards. The best success has been with large numbers of droplets with high spore content per droplet (Goettel et al. 2000).

Introducing large amounts of fungal inoculum into the soil and securing an even spread remains a problem. Many methods have been tested for application of fungal containing granules or conidia on grains to soil, including using seed drills for subsurface application, and hand application. The problems of spread of conidia after application to soil has led to the *Melolontha* and researchers are developing an area wide approach based on augmentative applications of *Beauveria brongniartii* for long term suppression of pest populations (Hajek et al. 2001).

2.3 Novel Strategies for Biopesticide Use

In some cases, preexisting application technology may not be well suited to the requirements of a biological agent. One

approach that takes advantage of the biological nature of entomopathogenic fungi is the "lure and infect" approach, best demonstrated by research on *Z. radicans* for control of diamondback moth. Furlong et al. (1995) have shown that using pheromone lures to attract moths to traps containing sporulating *Z. radicans* can result in contamination and spread of the fungus through the target population. Such an approach has been investigated for use with scarab beetles in the Azores (Klein and Lacey 1999). Autodissemination of entomopathogenic fungi for control of *Popillia japonica* in the Azores used a trapping system of commercially available attractants with *M. anisopliae*. The viability of conidia in traps after 6 days was found to be about 35%, but the basic process was successful for introducing fungi into pest populations. Another approach has been bait stations, such as those used with termites (Rath 2000). The entomopathogenic fungus is placed in a trap together with a food-based bait, and the insect becomes contaminated when it enters the trap. The general approach is similar for lure-and-infect and bait stations: attract the insect to an inoculum source, rather than broadcast application to secure contact between pest and disease.

Use of attractants is not restricted to luring to a single trap. Smith et al. (1999) investigated the use of vegetable fat pellets formulated with pheromone and *B. bassiana* to control the larger grain borer, *Prostephanus truncatus*. Significantly higher numbers of beetles were attracted to pellets containing pheromone than those without pheromone incorporated. The pellets containing pheromone and fungus could be stored for several weeks, indicating this may be a useful strategy to increase the utility of entomopathogenic fungi.

Development of biopesticides for social insects has been problematic because the method by which social insects defend against disease is mainly behaviorally-based rather than biologically-based. For example, hymenopteran wasps such as *Vespula* spp. have well-developed hygienic behaviour which includes removing all suspected material from a nest before contamination of nestmates occurs. *Vespula* do not reuse nests and, therefore, disease in one season does not result in disease in another season. Behavioral defense against disease requires novel application and formulation methods for any chance of success for entomopathogenic fungi. Similarly, termites are highly susceptible to entomopathogenic fungi, including *M. anisopliae* and *B. bassiana* but many factors such as avoidance of conidia, the removal and burial of fungus-killed termites, together with defensive secretions and inhibitory components in termite frass (Rath 2000), and grooming to remove spores (Milner and Glare, unpublished observations) reduce field efficacy. Boucias et al. (1996) used a low sublethal dose of a neurotoxin, imidacloprid to disrupt the grooming behaviour of termites, which then became highly susceptible to the fungus *B. bassiana*.

One proposal is to use more than one pathogen to increase the utility of entomopathogenic fungi. It is often common in the field to find more than one pathogen exerting influence on a pest, such as both a nucleopolyhedrovirus and the fungus *Entomophaga maimaiga* infecting gypsy moth (Malakar et al.

1999). The possibility of combining multiple species or strains in a single biopesticide to overcome limitations inherent in the single strain approach is intriguing. For example, Inglis et al. (1997) have investigated the use of both *M. anisopliae* and *B. bassiana* for control of locusts and grasshoppers, to overcome the temperature limitations of both species. There are potentially many methods whereby the efficacy of biopesticides could be enhanced by combinations, such as those described earlier, but the economics of producing multiple pathogens for a single product are usually too limiting.

3 BIOACTIVES FROM ENTOMOPATHOGENIC FUNGI

While the focus on the practical use of entomopathogenic fungi has been on biocontrol using whole organisms, either as inoculative or inundative agents (Eilenberg et al. 2001), these fungi are known to produce a number of toxins and enzymes. Some of these extracellular metabolites have been studied with the aim of using them as bioactives against insect pests. This biotechnological approach to utilizing entomopathogenic fungi can be demonstrated by the discovery and formulation of spinosyns, insecticidal toxins produced by an actinomycete. From the discovery of a strain of *Saccaropolyspora spinosa* in the Caribbean, Dow Agrow Sciences have successfully developed a number of "green chemistry" insecticide products, such as Success™ and Naturalyte™. It may be possible to utilize active components from entomopathogenic fungi in a similar or novel fashion.

It is not surprising that entomopathogenic fungi produce extracellular enzymes and toxins. These compounds are required to both assist penetration of the host cuticle and overcome other host defenses, while excluding competing microbes. Proteases produced by entomopathogenic fungi to degrade cuticle and assist entry into the host are similar to proteases used by insects to degrade their own cuticle during molting (Samuels and Paterson 1995). A number of enzymes are known from entomopathogenic fungi, such as the proteases, lipases, and chitinases that assist in cuticular breakdown. These enzymes can be thought of as bioactives and there has been increasing interest in use of these enzymes in pest control. Screen and St Leger (2000) have reported on the occurrence of trypsin and chymotrypsins in *M. anisopliae*. The novel chymotrypsin (CHY1) is similar to bacterial chymotrypsins. Because paralogous genes for the chymotrypsins are not found in genome sequences for yeast, gram eubacteria, archaeobacteria, and mitochondria they hypothesize that *chy1* arose from horizontal gene transfer.

Entomopathogenic fungi also produce insecticidal toxins. The early literature on toxins from entomopathogenic fungi was reviewed by Roberts (1981) and more recently by Strasser et al. (2000). Several metabolites from entomopathogenic Deuteromycetes are well known and described. For example, *Beauveria* spp. are known to produce beauvericin, a

depsipeptide metabolite which has shown toxicity to a number of invertebrates (Roberts 1981). Not all *Beauveria* can produce beauvericin, but it has been isolated from *Paecilomyces fumosoroseus* mycelium. *B. bassiana* is also reported to produce beauverolides, isarolides, and bassianolides, all cyclotetradepsipetides. *Metarhizium* strains are also well known for producing toxic metabolites, the best described of these are the destruxins. These cyclodepsipeptides are toxic to a number of insects, but susceptibility varies considerably, ranging up to 30 times between silkworm larvae and *Galleria* (Roberts 1981). Hirsutellin A is produced by *Hirsutella thompsonii* and is not proteolytic, but was toxic to a range of insects (Mazet and Vey 1995). *Aspergillus* species are occasionally insect pathogens and are known to produce many insecticidal metabolites. However, the occurrence of aflatoxin production in many *Aspergillus* that infect insects has restricted interest in this group, although it is by no means necessary that insecticidal strains produce aflatoxins in any appreciable amount (Roberts 1981). Not all entomopathogenic fungi produce toxins in the disease process. In some cases, toxins are suspected, but not conclusively demonstrated. Injection of culture filtrates of some entomopathogenic Entomophthorales into *Galleria* sp. resulted in blackening similar to that found in fully infected larvae [e.g., Roberts (1981)]. Some of the lower fungi, such as *Coelomomyces* and the Entomophthorales, may possess only weak toxins, if any at all. It is more likely they overcome hosts by utilizing the nutrients and invading vital tissue (Roberts 1981).

Some entomogenous fungi produce antibiotics. As entomopathogenic fungi must compete for utilization of cadavers with numerous resident and environmental bacteria, it is not surprising that a number of antibiotics are produced by the various strains and species. *Hirsutella* and the allied genus *Cordyceps* also produce a number of metabolites that may be weak toxins or antibiotics. Krasnoff and Gupta (1994) described an antibiotic, phomalactone, from the *H. thompsonii* var. *synnematosus* that was also toxic to apple maggots, *Rhagoletis pomonella* (Dipt., Tephritidae). Phomalactone was inhibitory to other entomopathogenic fungi (*Beauveria*, *Tolypocladium*, and *Metarhizium*). *Cordyceps*-infected caterpillars are a traditional medicine in parts of Asia. This may be partly based on the production by *Cordyceps* of a weak antibiotic, cordycepin. Zabra et al. (1996) reported that metabolites from *Z. neoaphidis* had antibacterial activity. In the future, bioactives from entomopathogenic fungi may have a role in pest insect control, either formulated as pesticides, or through transgenic expression. Direct toxicity may not be the only aim, as some toxins or metabolites have antifeedant type activities (e.g., <http://www.item.ba.cnr.it/biopesti.htm>).

4 MOLECULAR GENETICS OF ENTOMOPATHOGENIC FUNGI

The use of molecular techniques to manipulate entomopathogenic fungi to overcome some of the limitations discussed

earlier has been proposed for many years. In comparison with advances made in manipulation of viruses and bacteria, progress with the fungi has been slow, which is not surprising given the multigene nature of fungal insect diseases. Most progress has been made with the Deuteromycete muscardine fungi, *B. bassiana* and *M. anisopliae*.

4.1 Transformation Systems

Modification of entomopathogenic fungi has long been contemplated, but rarely reported. A limited number of studies have reported successful insertion of foreign genes into entomopathogenic fungi. A precursor to manipulation of entomopathogenic fungi using molecular techniques has been the development of transformation systems. There are several aims of transforming entomopathogenic fungi. These techniques enable gene disruption methods to be applied, which can lead to greater understanding of the genetics of disease processes, or the ability to introduce DNA into fungi may allow the modification of cell processes, potentially allowing improvements in the use of entomopathogenic fungi for insect control.

The first transformation of an entomopathogenic fungus was reported by Goettel et al. (1990), where *M. anisopliae* was transformed to be benomyl tolerant using pBENA3, a plasmid containing the *benA3* allele from *Aspergillus nidulans*. Since then, there have been other reports on transformation of the Deuteromycete entomopathogens using a variety of methods. St Leger et al. (1995) used electroporation and biolistic delivery to transform *M. anisopliae* with the plasmids (pNOM102 and pBENA3) containing the β -glucuronidase and benomyl resistance genes. The cotransformants showed normal growth rates and retained their pathogenicity to insects (*Bombyx mori*). Polyethylene glycol (PEG)-mediated transformation of protoplasts is another method for transformation of entomopathogenic fungi, as used with the *P. fumosoroseus* and *P. lilacinus* (Inglis et al. 1992) using benomyl as the selective agent. More recently, a heterologous transformation system for *B. bassiana* and *M. anisopliae* was developed based on the use of the *A. nidulans* nitrate reductase gene (*niaD*) (Sandhu et al. 2001). The *niaD* stable mutants of *B. bassiana* and *M. anisopliae* were selected by treatment of protoplasts with ethane methane sulfonate (EMS) and regenerated on chlorate medium.

4.2 Strain Improvement Through Biotechnology

Improvements in strains of entomopathogenic fungi have been attempted through selection as well as molecular methods. Selection of fungal strains with altered acyclic sugar alcohol (polyol) and trehalose content of the conidia may improve the endogenous reserves to enhance viability and desiccation tolerance. Cultures of *B. bassiana*, *M. anisopliae*, and *P. farinosus* grown under different conditions to obtain

conidia with a modified polyol and trehalose content resulted in conidia with increased intracellular levels of glycerol and erythritol that germinated more quickly than unselected conidia and at lower water activity (Hallsworth and Magan 1995). Conidia with increased trehalose germinated more slowly but stored for longer than unselected conidia. Another approach is to use genetic modification to “improve” strains and overcome limitations. This type of approach is in its infancy for entomopathogenic fungi, but there have been some interesting studies indicating the utility of the process. Two of the more promising studies on the potential of biotechnology to improve entomopathogenic fungi were published by Couteaudier et al. (1996) and Vaiud et al. (1998). They demonstrated that protoplast fusion between a strain of *B. bassiana* from *Leptinotarsa decemlineata* with an insecticidal toxin-producing strain of *B. sulfurescens* resulted in recovery of some di-auxotrophic mutants with enhanced activity (faster kill) against *L. decemlineata* and the caterpillar *Ostrinia nubilalis*. The stability of the virulence following passage through the insect–host and stability of molecular structure for two of the fusion products suggested that asexual genetic recombination by protoplast fusion may provide an attractive method for the genetic improvement of biocontrol efficiency in entomopathogenic fungi (Vaiud et al. 1998).

The most studied genes in the entomopathogenic fungi are the protease genes of *M. anisopliae*, particularly the *Pr1* gene. This was the first protease gene from an entomopathogenic fungi implicated in disease and was isolated by St Leger et al. (1992). *Pr1* has sequence similarity to proteinase K, but was more effective than that enzyme at degrading cuticle. It is similar to the subtilisin subclass of serine endopeptidases. Modification of *pr1* gene expression in *M. anisopliae* resulted in melanisation and cessation of feeding 25–30 h earlier than wild-type disease in caterpillars (St Leger et al. 1996). *V. lecanii*, *B. bassiana*, *Tolypocladium niveum*, and *P. farinosus* also produced *Pr1*-type enzymes during nutrient deprivation (St Leger et al. 1991). Southern analysis demonstrated that genes with significant homologies to *Metarhizium pr1* were present in the entomopathogens *A. flavus* and *V. lecanii* but not *Z. radicans* (St Leger et al. 1992). More recently, 11 subtilisin proteases (*Pr1*s) were identified from one strain of *M. anisopliae* (St Leger et al. 2001). Recently, intended field release of a modified *M. anisopliae* strain was reported (St Leger 2001). The strain has the *pr1* cuticle degrading protease gene under control of a constitutive promoter. The gene overproduction did not alter the host range, but resulted in a strain with a reduced median lethal time to kill. It also reduced the ability of transformants to sporulation. The *pr1* gene expression was under dual control of a general carbon catabolite repression/depression mechanism and a carbon source induction mechanism to control expression. Overexpression of extracellular chitinase, an enzyme important in the cuticular penetration of insects by entomopathogenic fungi, has also been demonstrated for *M. anisopliae* var. *anisopliae* (Screen et al. 2001). They expressed a chitinase gene from *M. anisopliae* var. *acridium* under control of an *Aspergillus* regulatory element to express

in noninducible conditions. While successful expression was achieved, there was no altered virulence to the caterpillar, *Manduca sexta*, compared to the wildtype fungus. Genetic manipulation of entomopathogenic fungi has a long way to go before transgenic pest control strains become available, if such technology is ever acceptable to regulators and the community. However, strain modification continues to provide a wealth of data on disease processes.

5 MOLECULAR IDENTIFICATION AND TRACKING

In addition to improving our understanding of the genetics of disease caused by entomopathogenic fungi, molecular techniques have also been used to aid in identification, classification, and environmental monitoring of fungi. It is now almost a standard practice to perform some form of genetic characterization of fungi to specifically identify strains used for biocontrol purposes. The utility of molecular techniques, however, has been demonstrated beyond simple strain identification. There are a number of reviews on the use of molecular characterization for entomogenous fungi in the literature [e.g., Driver et al. (1998) and Glare (2002)]. An example of the importance of molecular characterization was the clarification of the taxonomic position of *M. anisopliae* strain IMI 330189 used in the LUBILOSA program for locust control. Often described as a *M. flavoviride* strain because of the morphology of the conidia and some other features, the correct classification was debated. Recently, Driver et al. (2000) published a revision of the subspecific relationships between *M. anisopliae* and *M. flavoviride* strains, based largely on the sequence of the ITS-5.8s regions of rRNA. They demonstrated that IMI 330189 and related strains formed a discrete clade on the *M. anisopliae* branch of the *Metarhizium* trees, a finding that appears to have been well received by those working on locust control. Driver et al. (2000) named the subspecies *M. anisopliae* var. *acridium*, as well as describing several other subspecies, some of which can only presently be distinguished by ITS sequencing. This demonstrates a problem with molecular characterization, as fungal species cannot be solely erected on sequence data and requires supporting morphological or biological descriptions.

Molecular markers have been used to characterize the genotypes of individual fungal strains by examining gene products, but new techniques allow direct examination of variability at the DNA level. Pulsed field gel electrophoresis has been used to study karyotype variation in other fungi, and could be used with entomopathogens. Several studies have examined the number of chromosomes and mapped genes on those chromosomes. Viaud et al. (1996) studied the level of chromosome length polymorphism among nine isolates of *B. bassiana* to obtain a more extensive knowledge of the genomic organization. While extensive use of molecular characterization has proved useful, there are currently no standard techniques or agreement on even how many regions of the genome should be sampled to provide taxonomic data.

While this is not a problem for strain identification or comparison, it reduces the ability to compare between studies. Many of the molecular studies on entomogenous fungi have used the nuclear ribosomal DNA, but there are a number of other DNA regions used, such as mitochondrial DNA (mtDNA) restriction fragment length polymorphisms. The MtDNA has been used to estimate intraspecies variation in *V. lecanii* and *M. anisopliae* isolates.

The contribution of molecular techniques to the development of entomopathogenic fungi has been enormous. The techniques have been used to clarify evolutionary relationships [e.g., Driver et al. (2000) and Jensen et al. (1998)]. Molecular techniques have also allowed development of theories of evolution around these often obligate pathogens. Generally, studies on the entomogenous fungi using conserved mitochondrial or nuclear regions have failed to find a link between fungal species and host species. For example, Bidochka et al. (2001) found that habitat rather than host selection drives population structure of *M. anisopliae*. There have been exceptions, such as *B. bassiana* strains from *Sitona* weevils (Maurer et al. 1997) and some Entomophthorales [e.g., Jensen and Eilenberg (2001)]. In the order Entomophthorales, sequencing of the small subunit rDNA has been used to examine phylogenetic relationships (Jensen et al. 1998). The molecular studies supported the use of spore discharge characteristics as an identifying characteristic for Entomophthorales. The role of horizontal gene transfer in microbial evolution has been the topic and studies by St Leger et al. (2001) have found some evidence for the involvement of horizontal gene transfer in evolution of fungal parasitism, finding similarity between genes in *M. anisopliae* and *Streptomyces* bacteria. Monitoring of specific strains of entomopathogenic fungi in the field after release is crucial for advancing and understanding of biopesticide ecology. It has often been difficult to conduct ecological studies on fungal persistence and spread after application, because there has been a lack of simple methods for isolation and specific strain characterization of these fungi. The molecular characterization of strains of entomogenous fungi has improved the ability to track specific fungi in the field. Specific identification of the *B. brongniartii* strain used for control of the scarab pest, *Hoplochelus marginalis*, in the ReUnion Islands was based on introns (insertions) in the 28s gene of the rDNA (Neuvéglise et al. 1997). Genetic modification is also a method to allow tracking following release of a strain into the environment or in a host. For example, a β -glucuronidase gene has been inserted in *M. anisopliae* to allow detection of hyphae in infected hosts (St Leger et al. 1995) and the expression of a green fluorescent protein-encoding gene for tracking purposes (St Leger 2001).

6 SAFETY OF ENTOMOPATHOGENIC FUNGI

Biopesticides based on entomopathogenic fungi are now available, with a range of different species and strains used.

The growing use of these insect pathogens has raised interest in the safety of microbial pesticides, above the level previously required for registration purposes. This increased scrutiny of environmental and mammalian safety of entomopathogenic fungi is part of a worldwide move to more awareness of potential negative impacts of biotechnology. Few of the entomopathogenic fungi are thought to pose a direct threat to human health. There are exceptions, such as the entomophthoralean fungus, *Conidiobolus coronatus*, but it is unlikely any development of the potentially hazardous strains would be contemplated. Many products have required mammalian toxicology packages to be submitted during the registration process to demonstrate safety. Generally, the entomopathogenic Deuteromycetes are considered to have low risk of mammalian toxicity [e.g., Donovan-Peluso et al. (1980) and Shaddock et al. (1982)]. Recent papers on *Metarhizium* and *Beauveria* have raised some issues regarding mammalian safety of immunocompromised individuals (Burgner et al. 1998; Henke et al. 2002).

In addition to viewing bioactives from entomopathogenic fungi as potentially useful, there has been consideration of their effect as potential hazards in registering entomopathogenic fungi. There are some results showing activity against human cell lines, such as tumor cell lines for *P. tenuipes* cytotoxic components (Nam et al. 2001). Production of toxic secondary metabolites has caused problems in the registration of some fungi in Europe. Strasser et al. (2000) summarizes data on specific secondary metabolites (destruxins, efrapeptins, oosporein, beauvericin, and beauveriolides) produced by the genera *Beauveria*, *Metarhizium*, and *Tolypocladium*. They found that fungal bioactives posed no obvious risk to humans, although the number of detailed studies is limited. Some studies have indicated low-level activity against animals of selected bioactives such as destruxins of *Metarhizium* have an intraperitoneal injection LD₅₀ of 1–16 mg/kg in mice. However, the levels of metabolites produced during insect infection were much lower than in culture.

There is a growing body of research on nontarget impacts of fungal-based insecticides [e.g., Goettel et al. (2001) and Hokkanen and Hajek (2002)], which have not found increased environmental risk from their use. The present evidence is that mycoinsecticides are very safe in production and use from both an environmental and mammalian toxicity viewpoint (Goettel et al. 2001). However, the formulations being developed require stringent testing to ensure their superior safety compared with comparable chemical pesticides (Moore and Prior 1993). While the fungi themselves have generally not been found to be a risk through testing and natural exposure, the development of novel formulations and strain combinations will require careful evaluation to ensure no unexpected effects occur. This could be especially true of nontarget impacts. Similarly, any development of genetically modified strains will have to be carefully studied for environmental and mammalian safety. Regulations in all countries are becoming more stringent on these issues, especially for genetically modified organisms.

7 CONCLUSIONS

Biotechnological approaches to the study and development of entomopathogenic fungi have advanced the field in recent years. Improvements in formulations allowing new biopesticides to succeed in unexpected conditions (such as locust control in Africa), strain selection, and identification have advanced not only biopesticide formulation, but understanding of disease processes and ecology. More specific identification systems have allowed better monitoring of biopesticide applications as well as development of phylogenetic classification. Despite some success, commercial use of entomopathogenic fungi is restricted by high cost, inadequate or inconsistent efficacy, limited mass production capability, and poor shelf life. However, entomopathogenic fungi have several advantages over other microbes for formulation in biopesticides as many species have a robust spore stage, capable of survival in products for many months or years. Some are easy to grow on simple media and can be formulated using a number of simple procedures. They can often kill more than one target pest, although limited in host range enough for registration purposes. With continuing improvements in formulation and application technology, it is likely that many more niche biopesticides will come to market, especially with the increased markets due to a rise in organic production and the reduction in the number of chemical pesticides available.

There are a number of new techniques and applications that will aid in the further development of entomopathogenic fungi. Application of molecular biological techniques to entomopathogenic fungi also holds the promise of strain improvement through genetic manipulation, or assist in strain improvement without genetic modification. For example, through techniques such as protoplast fusion and chromosome exchange, using knowledge of desired chromosomal gene location, may enable superior strain qualities to be combined in single isolates. Determining the underlying genetics of host specificity, the toxins and enzymes involved in the disease process, and genetics of fungal processes such as sporulation and germination are all under study around the world. Advances in these areas may allow greater use to be made of entomopathogenic fungi.

The potential of entomopathogenic fungi lies not just in their application as biopesticides based on the live fungus, but also in the isolation and development of bioactives from these fungi. Toxins, enzymes, and antibiotics are all produced by entomopathogenic fungi and, as techniques for their isolation and expression increase, the potential for exploiting bioactives is enhanced. In some cases these bioactives are not toxins, but may exert other useful effects, such as antifeeding activity. There is also potential in novel strategies for biopesticide use such as mixtures of behaviour-modifying chemicals for enhancing control of social insects with pathogens.

While the history of biopesticide development from entomopathogenic fungi is littered with more failures than

success, the future seems brighter. As the knowledge from several commercially successful products and new technologies are applied to biopesticide development, we can expect to see more novel biocontrol methods applied to insect pests in the future using fungal species.

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Biotechnological Potential of Ergot Alkaloids

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1 INTRODUCTION

Ergot alkaloids belong to the group of compounds produced by fungi, which are referred to as secondary metabolites. They are produced by a number of fungi mainly of *Claviceps* spp. but they have been also found in other fungi and higher plants. Ergot (sclerotium of the pyrenomycete *Claviceps purpurea*) develops in florets of grasses and sedges. In early days, the medieval midwives used to collect the fungus from naturally infected plants and used it in the induction of childbirth and in the control of postpartum bleeding. The role of ergot has undergone important changes from a dreaded toxic parasite to an important source of biologically effective substances. *C. purpurea* is, apart from yeast, the first fungus, which was biotechnologically exploited without its existence known. The beginning of modern ergot research dates back to the extraction of the first alkaloid mixture from sclerotia in 1875, isolation of ergotoxine (mixture of ergocornine, ergocristine, and ergokryptine) in 1907, and the discovery of the first clinically used compound, ergotamine in 1918. At the beginning of fifties the chemistry, biosynthesis, physiology, biochemistry, genetics, biotechnology, and therapeutical applications of ergot alkaloids have been extensively studied (Berde and Sturmer 1978; Mukherjee and Menge 2000; Rehacek and Mehta 1993; Tudzynski et al. 2001). The present review gives an overview of biotechnological potential of ergot alkaloids with a perspective for the future.

2 SOURCES

In nature, the ergot alkaloids are formed primarily by various species of *Claviceps*. However, ergot alkaloids have also been

recorded from outside this genus (Flieger et al. 1997; Kozlovsky 1999).

Ascomycetes

Eurotiales: *Aspergillus fumigatus*, *A. flavus*, *A. japonicus*, *A. tamarii*, *A. versicolor*, *A. nidulans*, *A. oryzae*; *Penicillium aurantiovirens*, *P. camembertii*, *P. chermesinum*, *P. fumigatus*, *P. clavigerum*, *P. concavorugulosum*, *P. crustosum*, *P. griseofulvum*, *P. kapuscinskii*, *P. palitans*, *P. patulum*, *P. roqueforti*, *P. rubrum*, *P. rugulosum*, *P. sizovae*, *P. viridicatum*

Hypocreales: *Balansia claviceps*, *B. epichloe*, *B. obtecta*, *B. strangulans*, *Epichloe typhina*, *Neotyphodium coenophialum*, *N. lolii*, *Hypomyces aurantius*, *Sepedonium* sp.

Basidiomycetes *Corticium caeruleum*, *Lenzites trabea*, *Pellicularia filamentosa*

Zygomycetes *Cunninghamella blakesleana*, *Mucor hiemalis*, *Rhizopus arrhizus*, *R. nigricans*

Higher plants

Convolvulaceae *Argyreia nervosa*, *Ipomoea argyrophylla*, *I. rubro-coerulea*, *I. piurensis*, *Rivea corymbosa*, *Stictocardia tiliifolia*

3 STRUCTURE

The ergot alkaloids constitute the largest known group of nitrogenous fungal metabolites and over 80 alkaloids have been isolated from diverse natural material. The common part of chemical structure of the most ergot alkaloids is a tetracyclic ergoline ring system (Figure 1), which is biosynthesized from tryptophan (Taber and Vining 1959), and mevalonic acid (Groger et al. 1961). The ergot alkaloids

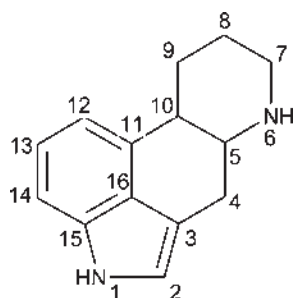


Figure 1 Structure of ergoline.

can be separated into three main structural groups: (a) clavine alkaloids, (b) simple lysergic and paspalic acid derivatives, and (c) peptide alkaloids (ergopeptines). The clavine alkaloids are tricyclic (secoergolines) or tetracyclic (ergolines) compounds usually substituted with methyl, hydroxyl or hydroxymethyl group in position C-8 and in many cases have a double bond in positions 8,9 ($\Delta^{8,9}$ -ergolines) or 9,10 ($\Delta^{9,10}$ -ergolines) of the ergoline skeleton (Figure 2). The important feature of all $\Delta^{9,10}$ -ergolines (including simple derivatives of lysergic acid and ergopeptines) is an easy isomerization on C(8) resulting in formation of two isomers. Clavine alkaloids represent the largest group of ergot alkaloids due to the action of various enzymes, which direct agroclavine and/or elymoclavine from the main biosynthetic route agroclavine–elymoclavine–paspalic acid–lysergic acid. Such type of shunt activity is particularly evident in microorganisms that lack the complete pathway and are unable to synthesize substituted lysergic acid (Vining 1980).

The simple derivatives of paspalic and lysergic acid (Figure 3) are mostly amides, in which the amide part is either small peptide or a simple alkylamide. Amides of lysergic and paspalic acids found in ergot are ergometrine, lysergic acid 2-hydroxyethylamide, lysergic acid amide (ergine), paspalic acid, and 10-hydroxy-paspalamide (Flieger et al. 1993). Ergopeptines are derivatives of lysergic acid and are the only

natural substances having the unique structure called cyclol (Figure 4) that consists of three aminoacids. The ergopeptines are further divided to (a) ergotamine, (b) ergoxine, (c) ergotoxine, and (d) β -ergoannine group (Flieger et al. 1997).

4 BIOGENESIS

Ergot alkaloids are derived from tryptophan, mevalonic acid, and methionine (Birch et al. 1960; Groger et al. 1960; Taylor and Ramsted 1960). The origin of the ergoline part from tryptophan and dimethylallylpyrophosphate was established at an enzymic level. The cell free biosynthesis of chanoclavines-I and II, agroclavine, and elymoclavine from precursors has been reported by Sajdl and Rehacek (1975) and Cavender and Anderson (1970). Tryptophan has also been found to be a factor in the induction and depression of enzymes catalyzing alkaloid formation (Krupinski et al. 1976). The first enzyme of alkaloid biosynthesis is dimethylallyltryptophan synthase (DMATS) (Heinstein et al. 1971) and the encoding gene was cloned from *C. fusiformis* and *C. purpurea* (Tsai et al. 1995; Tudzynski et al. 1999). Recent data of Tudzynski et al. (2001) show that in *C. purpurea*, all genes involved in alkaloid biosynthesis are organized in a cluster and are regulated by phosphate and pH level. This finding verified the previously published data on the negative role of phosphate in the induction of enzymes catalyzing the alkaloid synthesis (Krupinski et al. 1976) and on inhibition of alkaloid synthesis by high concentration of phosphate in the culture medium (Mehta 1984; Pazoutova et al. 1983). Gene for peptide synthetase homologous to that of *C. purpurea* was also detected in *Epichloe* and *Neotyphodium* species (Annis and Panaccione 1998; Panaccione et al. 2001).

Ergot alkaloid synthesis requires changes in differentiation. In *C. purpurea* the formation of conidia is inversely dependent on the synthesis of alkaloids for those saprophytic strains, which partially retain parasitic development, i.e., differentiation of the sphacelial phase to the conidial or the

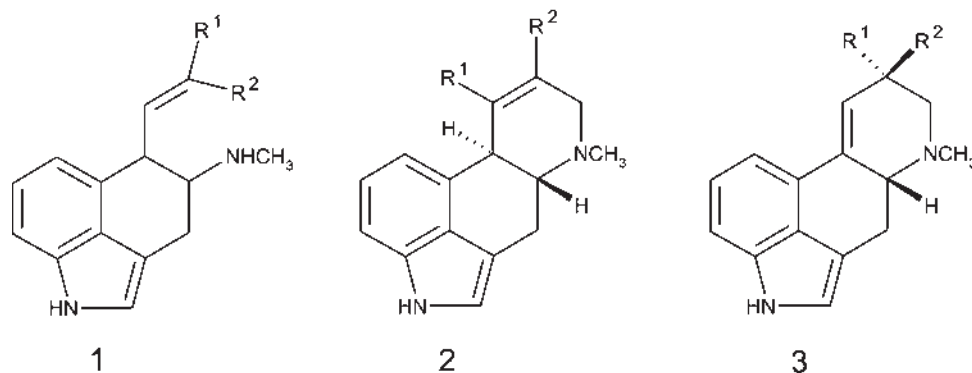


Figure 2 General structure of secoergolines (1), $\Delta^{8,9}$ -ergolines (2), and $\Delta^{9,10}$ -ergolines (3).

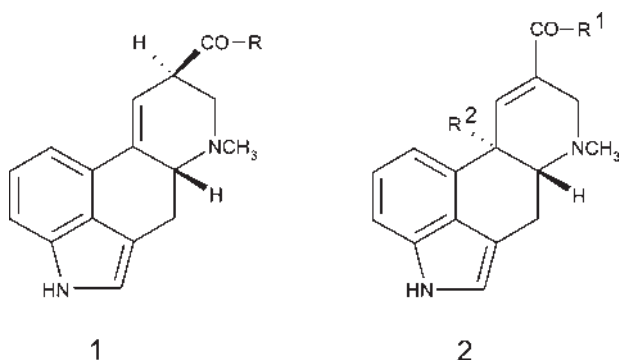


Figure 3 General structure of lysergic (1) and paspalic (2) acid derivatives.

sclerotial phase or both (Mehta 1984). In strains with not clearly separated conidial phase, vegetative growth, and the alkaloid phase, the conidia formation occurs simultaneously with alkaloid synthesis. The morphological development of the mycelium in submerged *Claviceps* cultures shows significant differences between high-yielding and degenerated cultures (Flieger et al. 1982). The character of fat hyphae and sclerotia-like cells of submerged mycelium is reminiscent of the plectenchymatic structure of parasitic cultures and have been found to influence the alkaloid production in *C. fusiformis* (Dickerson et al. 1970).

Cultivation conditions convenient for primary metabolism are not suitable for high alkaloid production (Taber and Vining 1963). To obtain overproduction of alkaloids, high citrate or Krebs cycle intermediate level in the medium is required (Pazoutova et al. 1981). The inability of submerged *Claviceps* cultures to grow on hexoses in absence of Krebs cycle intermediates comes from the parasitic way of life where the level of citrate and malate in the host plant phloem sap is high. The rate of oxidative metabolism of saccharides and the activity of alkaloid synthesis were proved to be proportional (Pazoutova et al. 1981). Another important fact is that clavine alkaloids are extracellular products with high solubility in the culture medium and feedbacks regulate their own biosynthesis (Flieger et al. 1988). Ergopeptines are mostly intracellular and are accumulated in lipid droplets (oleosomes) with no influence on the metabolism (Neumann et al. 1979).

5 INDUSTRIAL PRODUCTION

5.1 Industrial Production of Ergot Alkaloids

The overall world annual production of ergot alkaloids was estimated at about 20,000 kg (Cvak 1999) in which the production of ergopeptines and their dihydroderivatives forms less than one third. The rest of the production is

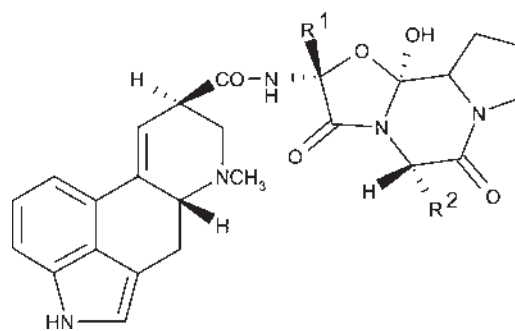


Figure 4 General structure of ergopeptines.

concentrated on production of lysergic acid and other precursors of semisynthetic ergot preparations (lysergol, elymoclavine, ergine, and other simple derivatives of lysergic acid). In the last decade the production of ergopeptines (Table 1) remained confined due to their limited therapeutic use while the production of semisynthetic ergot preparations is gaining importance due to the development of new drugs with new and more specific therapeutical applications (Berde and Sturmer 1978; Eich and Pertz 1999; Pertz and Eich 1999). It is evident from the data (Table 1) that lysergic acid is the main precursor of semisynthetic ergot preparations, which can be obtained by chemical decomposition of ergopeptines or simple derivatives of lysergic acid. So far, very limited amount of ergot preparations have been synthesized from clavine alkaloids. Field production of ergot alkaloids is still an important source of ergopeptines. In the last decades, the major effort was devoted to selection of strains producing defined spectrum of alkaloids. Recently, the average yield of ergot reached the level of 1000 kg/ha with content of alkaloids above 1% (Cvak 1999).

5.2 Saprophytic Cultivation of *Claviceps*

5.2.1 History

The first saprophytic cultivation of *Claviceps* on artificial nutritional media dates back to 1922 (Bonns 1922). The first attempt for the industrial production of ergot alkaloids was isolation of clavine alkaloids from submerged cultures of different *Claviceps* spp. (Abe and Yamatodani 1954; 1955; Abe et al. 1952; 1956). Later, conditions for saprophytic production of simple derivatives of lysergic acid were developed using different strains of *C. paspali* (Arcamone et al. 1960; 1961). It took only 5 years more when new isolate of *C. purpurea* was found to produce ergotamine under submerged conditions (Amici et al. 1966). Since that time all types of ergot alkaloids for direct use as therapeutic agents or precursors for the preparation of semisynthetic drugs can be obtained by fermentation.

Table 1 Annual production, therapeutic use, and source of recently used ergot preparations

Substance	Annual production (kg)	Therapeutic use	Source
Ergotamine	1000–1500	Uterotonic, antimigraine, vasoconstrictor, hemostatic	Field production, submerged production, synthetic
Ergometrine	100–200	Uterotonic, oxytocic	Field production, submerged production, synthetic
Dihydroergotamine	1500–2000	Antimigraine, sympatholitic, vasoconstrict	Hydrogenation of field/fermented ergotamine
Dihydrotoxin	1000–1500	Cerebral and peripheral vasodilator	Hydrogenation of field/fermented ergot mixtures
Dihydroergocristine	1000–1500	Sympatholitic, peripheral vasodilator	Hydrogenation of field/fermented ergocristine
Dihydro- α -ergokryptine	500	Antiparkinsonian, prolactine inhibitor, cerebral vasodilator	Hydrogenation of field/fermented α -ergokryptine
Bromokryptine	1000	Dopamine agonist, antiparkinsonian, prolactine inhibitor	Bromination of α -ergokryptine
Nicergoline	10000	Cerebral vasodilator	Synthesized from lysergic acid/lysergol
Metergoline	50	Serotonine antagonist, antimigraine, prolactine inhibitor	Synthesized from lysergic acid amide/dihydrolysergol
Methylergometrine	150	Uterotonic, oxytocic	Synthesized from lysergic acid
Methysergide	50	Serotonine antagonist, antimigraine	Synthesized from lysergic acid
Lisuride	30	Serotonine antagonist, prolactin inhibitor, antiparkinsonian	Synthesized from lysergic acid/ erginine
Terguride	10	Dopamine agonist, prolactine inhibitor, antiparkinsonian	Synthesized from lisuride
Pergolide	50	Dopamine agonist, prolactine inhibitor, antiparkinsonian	Synthesized from dihydrolysergol
Cabergoline	30	Dopamine agonist, prolactine inhibitor, antiparkinsonian	Synthesized from dihydrolysergic acid

5.2.2 Strain

As with other fermentation processes the key to successful production of ergot alkaloids is in obtaining the proper strain of the fungus. There are three main processes used for the preparation of *Claviceps* strains for saprophytic culture. (a) Plating of plectenchymatic tissue from the surface sterilized sclerotia on an agar growth medium (Mantle 1969), (b) Plating of honeydew drops containing conidia formed at early stage of *Claviceps* infection (Janardhanan and Husain 1984; Pazoutova et al. 2002), and (c) Trapping of sexual ascospores ejected from fruiting bodies on germinated sclerotia. By this method monosporic culture can be obtained (Vasarhelyi et al. 1980).

5.2.3 Strain Improvement

The classical methods (selection pressure, mutagenesis, and recombination) used for the strain improvement are, to some extent, more complicated with *Claviceps* due to incomplete information on cell nucleus. Strains used for saprophytic cultivation might be heterokaryotic and homokaryotic (Didek-Brumec et al. 1991; Mantle and Nisbet 1976). Recently it was found that the number of chromosomes in *C. purpurea* is variable so that haploid as well as aneuploid nuclei may be encountered (Hüsigen et al. 1999). Mutagenesis followed by subsequent selection of strain is an important technique in increasing the yield of alkaloids (Didek-Brumec et al. 1987). An ergocristine producing *C. purpurea* strain showed 180-fold increase in alkaloid production after eight-step mutation-selection with different mutagens (Kobel and Sanglier 1978). Mutagenesis of sporulating strain results in monosporic isolates. To increase the mutation frequency the protoplasts prepared from spores of selected strains were used (Olasz et al. 1982; Zalai et al. 1990). More complicated situation is with mutagenesis of asporogenic strains. Hyphal fragments are rather unsuitable for mutagenesis due to the higher number of nuclei. Even protoplast formation from young mycelium and subsequent regeneration without any mutagenic treatment yielded strains with different properties (Schumann et al. 1987). Protoplast fusion technique is beginning to find useful applications either in producing improved mutant strains by intraspecific crosses or in formation of novel spectrum of products by interspecific hybrids (Socic and Gaberc-Porekar 1992). Relevant structural and regulatory genes of the alkaloid biosynthesis in *C. purpurea* form a cluster of about 50 kbp in length (Tudzynski et al. 2001), therefore, isolation and cloning of the entire pathway to more rapidly growing fungus would be difficult.

5.2.4 Maintenance Improvement and Long-Term Preservation

Degeneration, loss of production capabilities, is a general problem of high-yielding strains of *Claviceps* (Kobel 1969). Conservation and systematically performed selection of the

isolates is the only way to eliminate the biological effects given by the transfer of cultures, ageing, and other influences. Methods applied for long-term preservation were recently reviewed (Hunter-Cervera and Belt 1996). For preservation of sporulating cultures two main methods are applied: deep-freezing and maintaining of cultures on rye grains or agar slants placed in refrigerator. Nonsporulating strains due to higher sensitivity to conservation procedure are frequently preserved as cultures on the agar plates. The universal technique applied for preservation is keeping of lyophilized cultures and cultures frozen, in liquid nitrogen (Baumert et al. 1979).

5.3 Fermentation

All technologies developed for industrial scale have the same aims: maximal production of desired ergot alkaloids, minimum amount of accompanying contaminants (other alkaloids, toxins, etc.), the shortest possible time of fermentation, minimized cost of medium, energy, equipments, and labor. For the production of ergot alkaloids, different fermentation technologies have been employed (a) stationary cultivation on liquid or solid medium (Kybal and Vlcek 1976; Trejo-Hernandez and Lonsane 1993), (b) submerged cultivations (Kobel and Sanglier 1986) also adapted to semicontinuous or continuous processes (Kopp and Rehm 1984). In some cases the immobilized microorganisms were used for production of ergot alkaloids under condition of submerged fermentation (Komel et al. 1985; Kopp and Rehm 1983; Kren 1991).

5.3.1 Stationary Surface Cultivation

In the beginning of sixties, the development of processes for production of ergot alkaloids under conditions of stationary cultivation on liquid media started (Adams 1962; Kybal et al. 1960; Molnar et al. 1964; Rochelmeyer 1965). The stationary surface cultivation on agar slants was commonly used for preparation of starting cultures. When transformed to industrial scale this technology showed some limitations mostly due to difficulties with control of aseptic conditions of large surfaces. Vlcek and Kybal (1974) developed technology for stationary cultivation of *C. purpurea* in plastic bags partially filled with inoculated liquid medium. This procedure was used for production of ergotoxins and later adapted to production of asexual spores of *C. purpurea* for field production.

5.3.2 Submerged Cultivation

Submerged fermentation in laboratory scale, i.e., shaker cultivation, is a primary step in getting knowledge of production microorganism physiology, biosynthesis, sporulation, stability, and influence of medium composition on production of alkaloids. In industrial scale, submerged fermentation in shaker culture is mostly used for preparation

of sufficient amount of inoculum for further cultivation step. During the inoculum preparation, an optimal state of the culture for biosynthesis of ergot alkaloids in the production step can be established (Socic et al. 1985; 1986). Usually, the whole industrial process consist of four steps i.e., (a) shaker culture, (b) preinoculating fermentation, (c) seed fermentation, and (d) production fermentation followed by downstream processing (Malinka 1999). Industrial production of clavine alkaloids, agroclavine, and elymoclavine by submerged fermentation of different strains of *Claviceps* sp. was reported in a number of patents (Kren et al. 1988; Rehacek et al. 1986; Trinn et al. 1990), reaching the maximum production of alkaloidal mixture about 6 g/l (Pazoutova and Tudzynski 1999; Pazoutova et al. 1987). In contrast to high production and well elaborated procedure of submerged cultivation the industrial production of clavine alkaloids does not receive much attention due to limited amount of procedures leading to preparation of desirable final products (cf. Table 1). Production of lysergic acid and its simple derivatives is of high technological and industrial interest due to relatively simple procedure of chemical modifications to semisynthetic ergot preparations. The main producers of these alkaloids are strains of *C. paspali*. The basic studies on biosynthesis, physiology, and production of simple derivatives of lysergic acid were done on relatively limited number of strains including *C. paspali* 31 (Rosazza et al. 1967), *C. paspali* ATTC 13892 (Socic et al. 1986), and *C. paspali* MG-6 (Bumbova-Linhartova et al. 1991). The concentration of produced alkaloids reached nearly 3 g/l of fermentation broth (Pertot et al. 1990) with the strain *C. paspali* L-52. One of the very interesting features of *C. paspali* strains is their ability to convert clavine alkaloids added to the cultivation medium to the simple derivatives of lysergic acid (Mothes et al. 1962). Flieger et al. (1989a, b) and Harazim and Malinka (1989) used this capability of *C. paspali* CCM 8061 and developed technology of aggressive bioconversion of clavine alkaloids to simple derivatives of lysergic acid with total production of nearly 6 g/l in batch cultivation and about 3 g/l in industrial fermentor.

Different strains of *C. purpurea*, as the only producers of ergopeptines, were described for their submerged production. Ergotamine producing strains and their cultivation are the best-studied processes due to direct therapeutical use of ergotamine. Industrial technologies were developed for the following strains: (a) F.I. 32/17 producing 2 g/l of ergotamine and α -ergokryptine mixture (Amici et al. 1966), (b) IBP 47, IMET PA135 producing 1.5 g/l [mixture of alkaloids containing 75% of ergotamine (Baumert et al. 1979)], (c) L-4 (ATTC 20103) producing 1.5 g/l of ergotamine (Komel et al. 1985). Another type of ergopeptines produced by submerged cultivation belongs to group of ergotoxines, i.e., ergocristine, ergocornine, and α -ergokryptine. Between many published procedures and industrial technologies (Malinka 1999) the process developed for strain *C. purpurea* L-17 resulted in relatively high production of ergotoxines (2.4 g/l). This strain was further studied and intermediary metabolism

and production of secondary metabolites were correlated (Gaberc-Porekar et al. 1992).

5.3.3 Solid Substrate Fermentation

Robinson et al. (2001) has recently proposed solid substrate fermentation (SSF) for the production of enzymes and secondary metabolites. The production of ergot alkaloids by *C. fusiformis* using SSF procedure was found to be 3.9 times higher than that obtained by submerged liquid fermentations (SLF) (Hernandez et al. 1993). One of the reasons could be the necessity of use of antifoam chemicals and the shear stresses caused by stirring in SLF. Also, better air circulation can be achieved in SSF thus further increasing the ergot alkaloid yields (Balakrishnan and Pandey 1996). The SSF has been shown to produce a more stable, requiring less energy in smaller fermentors with easier downstream processing measures. Also, by removal, the cost and trouble associated with antifoaming chemicals and by maximizing yield production, SSF may be seen as a viable option for industrial scale production of ergot alkaloids.

5.4 Optimization, Control, and Modeling of Ergot Alkaloids Fermentation

The following facts should be taken into account for optimization and scale-up of fermentation processes for the production of ergot alkaloids (Kobel and Sanglier 1978), (a) the production strains should be continually tested to maintain the optimal quality of selected production strain and thus maximally eliminate the biological effects given by transfer of cultures, ageing, and other external influences, (b) long cultivation period in absence of antibiotics require very high standard of sterility in operation and equipment, (c) balanced aeration and stirring are required due to the sensitivity of the *Claviceps* cultures to the stress and high oxygen tension, and (d) the use of antifoam agents could cause considerable loss in alkaloid yield. Recently the application of oxygen vectors to *C. purpurea* cultivation was published (Menge et al. 2001). The classical problem of the large-scale fermentations is an optimal supply of oxygen to growing *Claviceps* sp. High oxygen demand in the exponential growth phase can be met by addition of different hydrocarbons (Gilmanov et al. 1996) or perfluorocarbons (Menge et al. 2001) to shake flask and/or stirred reactors. Perfluorocarbons were successfully applied also in cultivations of other microorganisms (Lowe et al. 1998). Besides these technological aspects, other techniques to optimize the fermentation process were described. The influence of nutrients, addition of ergot alkaloid precursors, mainly tryptophan and its derivatives, were described in many studies (Erge et al. 1984; Floss 1976). As a result of feeding, prolonged idiophase of the fermentation process was found (Milicic et al. 1987) and this process seems to be one of the perspectives in modern alkaloid production (Socic and Gaberc-Porekar 1992). Precursor controlled production of

modified ergopeptines was described by Bianchi et al. (1982) and Crespi-Perellino et al. (1992).

The growth of the fungus and alkaloid formation in submerged batch fermentation was described by mathematical model, which can be further used in automatic process control and optimization. Grm et al. (1980) proposed for *C. purpurea* growth model based on morphological features of a cell population during the fermentation process. Votruba and Pazoutova (1981) proposed another model accentuating the antagonistic effects of phosphate on growth and alkaloid production. A mathematical simulation of different technological alternatives of clavine alkaloid production was done on this basis (Pazoutova et al. 1981). The activation–inhibition kinetics of clavine alkaloids production was evaluated for two *C. fusiformis* strains (Flieger et al. 1988) and it was found that feed-back inhibition can be eliminated by combination of fermentation and separation units in a closed loop. Increased efficiency (more than 100%) of the fermentation process was also found when inducers of cytochrome P-450 were used (Rylko et al. 1988).

6 CONCLUSIONS

The biotechnological relevance of ergot alkaloids is due to their therapeutical use, unquestionable. On the other hand, they play very important role as toxins in agricultural industry as products of endophytic fungi of the genus *Neotyphodium* and their production is coupled with serious problems of livestock grazing infected grasses. These two examples show the importance of molecular genetics of alkaloid biosynthesis. It could help, on one side, to develop new strategies for rational designing of ergot alkaloid based drugs, and, on the other one, to understand and control the production of ergot alkaloids by endophytic fungi. The following points need more attention for modern production of ergot alkaloids and its semisynthetic derivatives: (a) shortening of the initial nonproductive phase of alkaloid fermentation, (b) immobilization of cells, (c) construction of plasmids which can carry selectable markers, (d) the development of transformation systems with drug resistance markers, (e) study of membrane processes and vacuoles in the productive organism, (f) use of mathematical modeling for description of phenomenon observed during culture growth and alkaloid production, and (g) further development of solid state fermentation and application of oxygen vectors.

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Fungi as Plant Growth Promoter and Disease Suppressor

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1 INTRODUCTION

Reduction of the use of fertilizers and fungicides in agricultural production is necessary to help maintain ecosystems and to develop sustainable agriculture. The use of both bio-fertilizers and biocontrol systems can have minimal affect on the environment and such strategies have been widely researched. In soils, numerous microorganisms co-exist in association with plant roots. Some microorganisms live specifically in rhizosphere or on plant root surfaces, and these can have many effects on performance of the plant and may also affect the structure of the plant community. A unique microflora is particularly present around the plant root surface, where various substances are secreted. Most of the microorganisms distributed around plant root surface have a role in the decomposition of organic matter and some may suppress deleterious microorganisms, which could inhibit plant growth. Some of the root-associated microorganisms can promote plant growth, and they have been called “plant growth-promoting rhizobacteria” (PGPR; Kloepper et al. 1980) or “plant growth-promoting fungi” (PGPF; Hyakumachi 1994). The PGPR and PGPF are known to suppress some plant diseases. Similar effects are also observed in plants treated with mycorrhizal fungi, which have a symbiotic relationship with most plant species. Endophytes can also promote plant growth and these have recently been considered as potential biological control agents. In this chapter, fungi as known as PGPF, mycorrhizal fungi, and endophytic fungi, which act as plant growth promoters and disease suppressors are considered.

2 FUNGI AS PLANT GROWTH PROMOTER

2.1 PGPF

Many fungi isolated from soil can inhibit plant growth. Generally, the inhibition of plant growth is mostly caused by

plant-pathogenic fungi, however, some fungi that are not normally considered as pathogens can also inhibit plant growth. These fungi have been termed indefinite pathogenic fungi, and in one study, isolates of *Eupenicillium javanicum*, *Penicillium janthinellum*, *P. citreonigrum*, and *P. citrinum* obtained from roots of zinnia plant caused a 23–57% inhibition of the growth of the same plant (Yuen and Schroth 1986). Gamliel and Katan (1991) reported that almost all the fungi isolated from the rhizosphere and roots of tomato inhibited the growth of the plant. In contrast other soil-borne fungi, such as *Trichoderma* sp., *Rhizoctonia solani*, and others, can promote significant plant growth. Most of these PGPF have a high rhizosphere competence as a character. Because the genera found to be PGPF are common soil-borne fungi, there is a possibility that fungi having a similar role of PGPF exist widely in natural ecosystems. Some examples of plant growth promotion by PGPF are shown in Table 1. Most of these studies were quantified from the relative dry weights of root or above-ground part of treated plant seedlings with PGPF compared to nontreated ones over periods as short as 4 weeks. In some cases, significant growth promoting effects of PGPF were observed as increased yield of plants grown in fields over longer periods of 14 weeks or more (Shivanna et al. 1994).

2.1.1 PGPF in *Trichoderma*

Isolates of *Trichoderma harzianum* and *T. koningii* have been shown to enhance seedling emergence in tomato with increased shoot and root dry weights when compared to nontreated control plants (Table 1) (Windham et al. 1986). These species also gave rise to increased shoot and root dry weights in tobacco (Table 1) (Windham et al. 1986). Isolates of *T. viride* have been reported to increase tomato plant height (Windham et al. 1986). Chang et al. (1986) have shown that isolates of *T. harzianum* enhanced seedling emergence in chilli pepper and promoted growth of tomato, chilli pepper,

Table 1 Growth promotion on plants treated with plant growth-promoting fungi (PGPF)

Fungus	Crop	Growth promoting effect	References
<i>T. harzianum</i>	Tomato	2.1–2.8 times	Windham et al. (1986)
	Tobacco	7.9 times	Windham et al. (1986)
	Red pepper		Chang et al. (1986)
	Periwinkle		Chang et al. (1986)
<i>T. koningii</i>	Bentgrass	9.1 times	Hyakumachi (1994)
	Tomato	2.6–3.2 times	Windham et al. (1986)
	Tomato	5.1 times	Hyakumachi (1994)
	Tobacco	2.7 times	Windham et al. (1986)
	Ryegrass	4.4 times	Hyakumachi (1994)
<i>T. ciride</i>	Tomato		Windham et al. (1986)
Sterile black fungus	Wheat	40%	Speakman and Kruger (1984)
	Rye	40%	Speakman and Kruger (1984)
Sterile dark fungus	Wheat	30%	Narita and Suzui (1991)
Sterile red fungus	Wheat	10–60%	Dewan and Sivasithamparam (1989)
	Rye	10–60%	Dewan and Sivasithamparam (1989)
	Ryegrass	10–60%	Dewan and Sivasithamparam (1989)
<i>R. solani</i> AG4	Radish	13.4–19.8%	Sneh et al. (1986)
		28.4–36.0%	Sneh et al. (1986)
	Carrot	80.0–97.7%	Sneh et al. (1986)
		55.0–150.5%	Sneh et al. (1986)
	Lettuce	58.4%	Sneh et al. (1986)
	Cotton	28.7%	Sneh et al. (1986)
<i>R. nigricans</i>	Tomato	42%	Lindsey and Baker (1967)
<i>F. roseum</i>	Tomato	54%	Lindsey and Baker (1967)
<i>Phoma</i> sp.	Wheat	46–77%	Shivanna et al. (1994)
	Bean	23–25%	Shivanna et al. (1994)
		11–52%	Shivanna et al. (1994)
	Cucumber	2.1 times	Hyakumachi (unpublished)
		1.8 times	Hyakumachi (unpublished)

and cucumber. Isolates of *T. harzianum* have also been used to enhance flowering of periwinkle and to increase the number of flowers per plant in chrysanthemum.

2.1.2 PGPF in Mycelial Fungi

Plant growth promotion has been obtained from isolates of mycelial fungi that do not produce any spores. These unidentified fungi have been termed sterile black fungus (SBF), sterile dark fungus (SDF), and sterile red fungus (SRF), and have been isolated from corn roots, wheat roots, and wheat and rye grass roots, respectively. Unidentified isolates considered to be SBF (Speakman and Kruger 1984) and SDF (Narita and Suzuki 1991) were shown to increase shoot dry weight in wheat and similarly isolates termed to SRF increased shoot wet weight in wheat (Dewan and Sivasithamparam 1989). Growth promotion by these unidentified fungi has been reported in other plants and isolates of SBF have been reported to increase shoot dry weight in barley (Speakman and Kruger 1984). Fungi considered as SRF have been reported to promote plant growth of rye, brome grass, chick pea, lupine, medic, pea, ryegrass, and clover, all of which are used as typical rotation crops with wheat, and resulted in an increased shoot fresh weight (Dewan and Sivasithamparam 1989). The mycelial isolates used in these studies have not been identified, although isolates of SRF are thought to be Basidiomycetes because of the presence of clamp connection. Strains of SBF and SDF are easily isolated from herbal plants as well as woody plants and their relationships to endophytic fungi are being currently considered.

2.1.3 PGPF in *Rhizoctonia*

A particular nonpathogenic strain of *R. solani* has shown growth promotion and significantly increased yield for various crops in field experiments (Sneh et al. 1986). These included increased wet and dry weights of radish roots and carrot roots, and increased weights of cotton fiber and wheat grains. In similar experiments with potato, although increases were observed in shoot and tuber weight until 63–70 days after transplanting, there was no increase in yield at the time of harvest. Some isolates of binucleate *Rhizoctonia* have been found to be PGPF (Harris et al. 1993; Villajuan-Abgona et al. 1996).

2.1.4 Other PGPF

Isolates of *Rhizopus nigricans* and *Fusarium roseum* have been reported to increase shoot dry weight in tomato (Lindsey and Baker 1967). Soil conditions such as pH, water, nutrient and organic content, together with the presence of other micro-organisms are important considerations for the introduction of beneficial micro-organisms into soil. Hyakumachi (1994) reported the plant growth promotion effect of PGPF occurred in sterilized or nonsterilized nutrient-deficient and rich soils, potting soil, and most conspicuously in the

nutrient-deficient one. The effect of PGPF was also observed in soil that had been converted to nutrient-rich by amendment with NPK fertilizer.

The duration of the plant-growth promoting effect of PGPF in the treated plants is an important factor for the earlier application. Increased growth responses were observed in wheat treated with PGPF isolates during the seedling stage (2 weeks after sowing), vegetative stage (4 weeks), pre-flowering stage (10 weeks), and seed maturation stage (14 weeks) (Hyakumachi 1994; Shivanna et al. 1994). All of the isolates used increased plant height, and also significantly increased the ear-head length, weight, seed number, and biomass (Table 1) (Shivanna et al. 1994).

In order to develop applications, it is important to isolate PGPF strains that (a) have high affinity for plants and can colonize their rhizosphere, (b) show high levels of plant growth promotion, and (c) offer consistent performance in field trials. Although an isolate with a wide host range is an ideal candidate, there is a requirement for isolates that show high specific effectiveness with an individual host plant.

2.2 Mechanisms of Plant Growth Promotion by PGPF

Several hypotheses have been put forward for the mechanisms of plant growth promotion by PGPF, including (a) hormone production, (b) substrate degradation (mineralization), and (c) suppression of deleterious microorganisms.

2.2.1 Hormone Production

Culture filtrates of certain fungal species promote plant growth, due to the production of plant growth hormones by these fungi (Ram 1959). Growth promotion has been seen in some plants after treatment with mycelial exudates from PGPF strains of *Trichoderma* and SRF, and a gibberellin-like substance was reported to be involved (Gillespie-Sasse et al. 1991; Windham et al. 1986). Some strains of *Phoma* species have been found to produce abscisic acid, and this compound is also reported to promote plant growth. However, in general terms, these appear to have little relationship between the production of plant growth hormones and the ability of PGPF to promote plant growth.

2.2.2 Mineralization

Close relationships have been shown between the reduction of barley grain weight due to PGPF, the subsequent growth promotion effect of PGPF, and their cellulase and starch degradation activity (Hyakumachi 2000). Production of $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ in soil can also be increased by amendment with PGPF-infested barley grains.

The total amount of nitrogen in PGPF infected-barley grains remains the same despite which PGPF isolate is used, however, the amount of $\text{NH}_4\text{-N}$ varies depending on the isolate, with the highest level being seen in grains infected

with *Phoma*. The $\text{NH}_4\text{-N}$ levels later decreased in the order *Phoma* > *Fusarium* > *Penicillium* > *Trichoderma* > control. The amount of $\text{NH}_4\text{-N}$ was about 7.8 times higher in *Phoma* infested-barley grains than that of control (Hyakumachi 2000). Hyakumachi (2000) also demonstrated correlations between reduction of barley grain weight and cellulase activity, starch degradation activity of starch, and the dry weight of bentgrass. These results suggest that the mineralization of organic substrates by PGPF relates to the plant-growth promoting effect of those PGPF. The PGPF may therefore provide the plant with necessary mineral nutrients in an easily assimilating form.

2.2.3 Suppression of Deleterious Microorganisms

A remarkable plant growth promotion effect has been reported for field-grown cucumbers, and this was attributed to the suppression of indigenous pathogenic *Pythium* spp. in the soil by PGPF (Hyakumachi 1994). The suppression of deleterious microorganisms by PGPF may therefore be one of the mechanisms of plant-growth promotion.

2.3 Mycorrhiza

As a definition of mycorrhizae, Smith and Read (1997) proposed "a symbiosis in which an external mycelium of a fungus supplies soil derived nutrients to a plant root." Mycorrhizae are further divided into six types based on anatomical characteristics, which are: (a) arbuscular mycorrhizae (AM), (b) ectomycorrhizae, (c) orchid mycorrhizae, (d) ericoid mycorrhizae, (e) monotropoid mycorrhizae, and (f) arbutoid mycorrhizae. Some plants have requirement for mycorrhizae in order to complete their life cycle. Mycorrhizae may influence host plant survival in regeneration niches and mycorrhizae can also increase seed production, seed quality, and host and offspring vigor. Some types of mycorrhizae enhance host plant resistance against severe environmental conditions. The most widely studied and most-commonly encountered mycorrhizal systems are the ectomycorrhizae and arbuscular mycorrhizae.

2.3.1 Ectomycorrhizae

About 5000 fungi, Asco- and Basidiomycetes, are known to form ectomycorrhizal association with about 2000 species of woody plants (Kendrick and Berch 1985). Roughly 5% of the vascular plants are known to develop ectomycorrhizae and these associations are typically seen by the intercellular development of Hartig nets. Ectomycorrhizal fungi are known to enhance the uptake of water and nutrients by the host plant, and to promote plant growth. Growth effects have been observed in a broad range of forest trees, such as Douglas fir, pine, and eucalyptus, with ectomycorrhiza associations forming in nurseries and in the field. *Laccaria laccota*, *Pisolithus tinctorius*, *Suillus plorans*, *Hebeloma cylindrosporum*, and *H. crustuliniforme* have been used as soil

inoculations and their growth promoting performances were dependent on the host plant. Large increases in the growth of pines have been recorded in field experiments. For instance, 25–100% increases in growth have been reported for three pine species inoculated with *Pisolithus tinctorius* on five reforestation sites in the southern United States (Marx et al. 1977). Inoculation with *Paxillus involutus* has been associated with a marked increase in stem diameter and volume, especially with sessile oak at Bouxières where the volume almost doubled over 7 years (Garbaye and Churin 1997).

The increase in growth resulting from inoculation with mycorrhizal fungi has been attributed to improved nutrition of the host plant in most cases. Ectomycorrhizal fungi are able to absorb and accumulate phosphorus, nitrogen, potassium, and calcium in the fungal mantles more rapidly and for longer periods of time than nonmycorrhizal feeder roots. Ectomycorrhizal fungi improve the efficiency of phosphorus uptake principally through the development of extramatrical hyphae, which increase the absorptive surface and effective rooting density of the plant. Ectomycorrhiza are likely to enhance N uptake where the fungus and host plant differ in their capacity to absorb and assimilate $\text{NO}_3\text{-N}$. Mycorrhizal fungi generally have a preference for $\text{NH}_4\text{-N}$, although a number of species can also utilize $\text{NO}_3\text{-N}$ (Plassard et al. 1991).

2.3.2 Arbuscular Mycorrhiza

Arbuscular mycorrhizal (AM) associations are due to Glomales, an order of Zygomycetes (Morton and Benny 1990). The order consists of 7 genera, *Glomus*, *Entrophospora*, *Acaulospora*, *Archaeospora*, *Paraglomus*, *Gigaspora*, and *Scutellospora*. Arbuscular mycorrhizal fungi develop arbuscules or hyphal coils within host plant cortical cells, and have a wide host range including many agricultural and horticultural crops worldwide. Growth promotion has been seen in many AM-associated plants including maize (Baltruchat 1987), tomato (Mohandas 1987), asparagus (Pedersen et al. 1991), Boston fern (Ponton et al. 1990), and gerbera (Wang et al. 1993). Despite the morphological differences between ecto- and arbuscular mycorrhizae, there appear to be many common features in their growth-promoting effects. Arbuscular mycorrhizal fungi develop extraradical hyphae that grow into the surrounding soil, increasing the potential of the root system for nutrient and water absorption, and improving the soil structure for better aeration and water penetration. One of the mechanisms of growth promotion by AM fungi involves the transport of phosphorus by AM fungi from the soil to the plant. Direct measurements of phosphorus transfer by AM fungal hyphae have been made by Jakobsen (1994) and Schweiger et al. (1999). Colonization of roots by AM fungi modifies the growth response of the plant and increases supplies of phosphorus (Abbott et al. 1995), however, some studies have shown that effectiveness, in terms of plant growth promotion, is not related to the extent of host root colonization (Jensen

1982; Sanders and Fitter 1992). Efficient phosphorus uptake has been found to be more closely related to the quantity of mycelium partitioned into the extraradical phase of the fungi (Abbott and Robson 1985; Morin et al. 1994). Jakobsen et al. (2001) reported that the phosphorus transport capacity of AM fungi is related not only to colonization rate, but also to the transport character of AM fungi themselves. The AM fungi cause few changes to root morphology, but the physiology of the host plant may change significantly. Tissue concentrations of growth-regulating compounds and other chemical constituents change, photosynthetic rates increase, and the partitioning of photosynthate to shoots and roots changes (Bethlenfalvay 1992). Allen et al. (1980) demonstrated differences in cytokinin content between *Bouteloua gracilis* plants with and without associated *Glomus fasciculatus*. They also reported quantitative and qualitative changes in GA-like substances in the leaves and roots of AM-associated plants (Allen et al. 1982). Increases in auxin, cytokinin, GA and B-vitamin production have also been reported in plants associated with ectomycorrhizal fungi (Crafts and Miller 1974; Slankis 1973; Strzelczyk et al. 1977).

2.4 Endophyte

A widely accepted definition of an endophyte is; “endophytes symptomlessly colonize the living, internal tissues of their host, even though the endophyte may, after an incubation or latency period, cause disease” (Petrini 1991). This definition includes virtually any microbe that colonizes the internal tissues of plants. For example, some plant-pathogenic fungi, such as the smut fungi, can be defined as endophytes unless the plant shows symptoms after the infection (Stone et al. 2000). Endophytes are generally known to enhance plant tolerance to environmental stresses, damage from harmful insects, and diseases caused by pathogens and nematodes. There are a few studies on the plant-growth promoting effect of endophytic fungi. Yetes et al. (1997) observed a slight but significant increase in plant weight, shoot height, and shoot diameter in *Fusarium moniliforme*-infected plants, 28 days after planting compared to uninoculated control plants. *Pinus contorta* inoculated with *Phialocephala fortinii* increased uptake of phosphorus and nitrogen, that resulted in enhanced growth of inoculated plants compared with noninoculated plants (Jumpponen and Trappe 1998).

3 FUNGI AS DISEASE SUPPRESSOR

3.1 PGPF

Almost all the PGPF reported so far have shown a pronounced suppressive effect against soil-borne diseases. One example of this is the suppression by *Trichoderma harzianum* of damping-off disease on barley, cucumber, radish, and tomato caused by *Pythium ultimum* (Ahmad and Baker 1988). Nonpathogenic *R. solani* AG4 has been reported to suppress

damping-off disease caused by virulent *R. solani* and *R. zeae* by 76–94% on cotton, radish, and wheat. The sterile fungi, SBF, SDF, and SRF, have been shown to decrease the occurrence of take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Dewan and Sivasithamparam 1989; Narita and Suzuki 1991; Speakman and Kruger 1984). The PGPF isolated from zoysiagrass rhizosphere have been shown high suppressive ability against soil-borne diseases caused by *Pythium aphanidermatum*, *P. irregulare*, *R. solani*, *Sclerotium rolfsii*, *Fusarium oxysporum* f. sp. *melonis*, *F. o. f. sp. cucumerinum*, *G. graminis* var. *tritici* and *Cochliobolus sativus* (Hyakumachi 1994). When cucumber plants inoculated with PGPF isolates from zoysiagrass rhizosphere, disease suppression was observed against the air-borne pathogen, *Colletotrichum orbiculare* (Meera et al. 1994). In this work the PGPF were applied to plant roots and leaves were used for pathogen inoculation thereby the PGPF and pathogen were physically separated. The result therefore suggests that induced systemic resistance is involved as one of the mechanisms for disease suppression by PGPF. The PGPF isolates from zoysiagrass rhizosphere, *Trichoderma*, *Fusarium*, *Penicillium*, *Phoma*, and sterile fungi, all provided significantly protection to air-borne anthracnose caused by *C. orbiculare*, bacterial angular leaf spot caused by *Pseudomonas syringae* pv. *lacrymaus*, and soil-borne Fusarium wilt by *F. oxysporum* f. sp. *cucumeris* (Koike et al. 2001). In the case of Fusarium wilt, a split-root system was used to ensure physical separation of PGPF and pathogen, and to assess induced resistance.

3.2 Mycorrhizae

Initial evidence for the role of ectomycorrhizal fungi in disease suppression was provided by a number of field observations that showed mycorrhizal-associated seedlings or trees of both angiosperms and gymnosperms were more resistant to root pathogens than their nonmycorrhizal counterparts (Marx 1973). Ectomycorrhizal roots of various *Pinus* spp. and Sitka spruce (*Picea sitchensis*) seedlings were resistant to infection by a *Rhizoctonia* sp. that could readily infect nonmycorrhizal feeder roots (Levisohn 1954). Richard et al. (1971) suggested that the presence of ectomycorrhizal fungus, *Suillus granulatus* in the substratum completely prevented any negative effect of endophytic *Mycelium radialis-atrovirens* on *Picea mariana* seedlings. Hashimoto and Hyakumachi (2001) also suggested that the ectomycorrhizal fungi suppressed the deleterious effect of endophytic *M. radialis-atrovirens* on *Betula platyphylla* var. *japonica* seedlings.

Arbuscular mycorrhizae associations have been shown to reduce damage caused by soil-borne plant pathogens. Although few AM isolates have been fully studied, some appear to be more effective than others. Furthermore, the degree of protection varies with the pathogen involved, and can be modified by soil types and other environmental

conditions. Trotta et al. (1996) reported that the AM fungus, *Glomus mosseae*, reduced adventitious root necrosis and necrotic root apices caused by *Phytophthora nicotianae* var. *parasitica* by 63–89%. The AM associations are also known to limit the damage by bacterial pathogens and pathogenic root nematodes (Garcia-Garrido and Ocampo 1989; Hussey and Roncadori 1982), however, the results are not consistent.

3.3 Endophyte

The enhanced resistance for disease shown by some endophyte-infected plants is generally considered to result from the production of defense compounds by the endophyte-plant infection. An example of this is the inoculation of maize kernels by endophytic *Fusarium moniliforme*, which is reported to protect against infection by pathogenic *F. graminearum* (Van Wyck and Scholts 1988). Nonpathogenic, endophytic strains of *F. oxysporum* isolated from suppressive soils have been used as biological control agents for manage diseases caused by pathogenic *Fusarium* species on watermelon, cucumber, celery, and other crops (Larkin et al. 1996; Schneider 1984). In each case, these fungi were endophytes of the hosts they protected. Endophytic *Heteroconium chaetosprira* is reported to almost completely suppress clubroot formation in Chinese cabbage caused by *Plasmodiophora brassicae* (Narisawa et al. 1998).

4 MECHANISMS OF DISEASE SUPPRESSION BY FUNGI

4.1 Antagonism

Many reports have shown that the growth-promoting effect of PGPF is due to their ability to suppress harmful microorganisms in the soil. It is generally accepted that hyperparasitism, antibiosis, and competition are all involved in the antagonistic activities of PGPF. The mechanisms of disease suppression by PGPF isolated from zoysiagrass are shown in Table 2. The isolates of PGPF did not show hyperparasitism to other fungi. In some cases involving *Trichoderma* there was a relation between antibiotic activity and disease suppression, however in most cases, disease suppression was closely related with the ability to compete for infection courts or nutrient on the surface of the plant root.

The production of antagonistic substances is thought to be one of the mechanisms of protection provided by ectomycorrhizal fungi. As an example of this, antibacterial activities have been demonstrated for *Paxillus involutus* and *Hebeloma crustuliniforme* in pure culture (Marx 1973) and for *Cenococcum graniforme* in mycorrhizal symbiosis (Krywolap et al. 1964). The antibiotic effect of mycorrhizal fungi was attributed to the production of organic acids as demonstrated for *P. involutus* (Duchesne et al. 1989). Olsson et al. (1996) demonstrated that presence of the ectomycorrhizal mycelium decreased bacterial activities as

Table 2 Mechanisms of disease suppression against pathogenic fungi and bacteria by plant growth-promoting fungi (PGPF) isolated from zoysiagrass

Pathogenic fungi	PGPF	Hyperparasitism	Antibiosis	Competition	Induced resistance
<i>R. solani</i>	<i>T. harzianum</i>	—*	±	+	NT**
	<i>Phoma</i> sp.	—	—	+	NT
	<i>F. equiseti</i>	—	—	+	NT
<i>P. irregulare</i>	<i>T. harzianum</i>	—	±	+	NT
	<i>Phoma</i> sp.	—	—	+	NT
	<i>F. equiseti</i>	—	—	+	NT
<i>S. rolfsii</i>	<i>T. harzianum</i>	—	±	+	NT
	<i>Phoma</i> sp.	—	—	+	NT
	<i>F. equiseti</i>	—	—	+	NT
<i>F. oxysporum</i> f. sp. <i>cucumerinum</i>	<i>T. harzianum</i>	—	±	—	+
	<i>Phoma</i> sp.	—	—	+	+
	<i>F. equiseti</i>	—	—	—	+
<i>C. orbiculare</i>	<i>T. harzianum</i>	—	±	—	+
	<i>Phoma</i> sp.	—	—	—	+
	<i>F. equiseti</i>	—	—	—	+
<i>P. syringae</i> pv. <i>lachrimans</i>	<i>P. simplicissimum</i>	—	—	—	+
	<i>T. harzianum</i>	—	±	—	+
	<i>Phoma</i> sp.	—	—	—	+
	<i>F. equiseti</i>	—	—	—	+
	<i>P. simplicissimum</i>	—	—	—	+

* + /— : Effective/not effective; ** NT: Not tested.

measured by using the thymidine incorporation technique. Zak (1964) suggested that ectomycorrhizal fungi may: (a) utilize surplus carbohydrates in the root thereby reducing the amount of nutrients stimulatory to pathogens, (b) provide a physical barrier, i.e., the fungal mantle, to penetration by pathogen, (c) secrete antibiotics inhibitory to pathogens, and (d) support along with the root, a protective microbial rhizosphere population. Marx (1969) suggested that inhibitors produced by symbiotically infected host cortical cells may also have a function as inhibitors of the infection and spread of pathogen in ectomycorrhizal roots.

Competition for colonization sites, direct antibiosis, nutritional aspects, and plant defense reaction have all been considered as possible mechanisms in disease suppression by AM fungi (Azcon-Aguilar and Barea 1996). However, these mechanisms are still poorly understood.

Antifungal activities are thought to be involved in mechanisms of disease suppression by endophytic fungi. The endophytic fungi *Neotyphodium coenophialum* and *N. lolii* have been shown to form an inhibition zone under dual culture with the pathogenic fungi, *Colletotrichum graminicola*, *Rhizoctonia cerealis*, *R. zeae*, etc. (Siegel and Latch 1991). These results suggest that these endophytic fungi produce antifungal substances. Volatile compounds were collected from both endophyte-infected and endophyte-free tall fescue, and the sheath of endophyte-infected plants was found to produce high levels of 1-octen-3-ol, a characteristic fungal toxic volatile compound derived from lipid peroxidation in fungi, which was absent in endophyte-free plants (Yue et al. 2001). Hydroxamate siderophore synthesis by *P. fortinii*, a typical dark septate fungal endophyte, was reported by Bartholdy et al. (2001). Iron is an essential micronutrient for almost every organism and siderophore synthesis by *P. fortinii* may be expected to play a key role in iron nutrition for the plant, resulting in a lack of available iron for pathogens.

4.2 Induced Resistance

Induced-systemic resistance has been observed on cucumber plants treated with PGPF from zoysiagrass (Hyakumachi 1997; Koike et al. 2001; Meera et al. 1994). Almost all of these PGPF could induce resistance against anthracnose in cucumber. In contrast, Ishiba et al. (1981) reported that only 1.9–2.4% of the soil fungi isolated from cucumber rhizosphere, were able to induce systemic resistance against anthracnose in cucumber plants. Different types of PGPF have been isolated from all over the world and it would be interesting to know if any of these have as high performance of induced systemic resistance as PGPF isolated from zoysiagrass. Recently, induced systemic resistance caused by binucleate *Rhizoctonia* and *Trichoderma* has been reported in plants. Some isolates within these fungi have growth-promoting ability and others have been used as biological control agents. The induced resistance on cucumber caused by PGPF isolated from zoysiagrass can last as long as 9 weeks

under glass house conditions and for up to 6 weeks under field conditions (Meera et al. 1995). Lignin deposition is known as one of the mechanisms of induced systemic resistance (Hammaerschmidt and Kuć 1982). Koike et al. (2001) reported that lignification of cucumber seedling hypocotyls was induced by culture filtrates of PGPF, following challenge inoculation with *C. orbiculare*. The result showed enhanced lignin deposition in cucumber after infection by *C. orbiculare* as compared to the control. The elicitor activity of culture filtrates of PGPF has been evaluated by chemiluminescence to determine the emission of active oxygen species from tobacco callus and cucumber fruit disks (Koike et al. 2001). The oxidative burst is characterized by a rapid and transient generation of active oxygen species immediately following fungal elicitor treatment. From these results, the >12,000 MW fraction and both >12,000 MW fraction and lipid fraction from the culture filtrate elicited the highest superoxide generation, respectively. A high correlation between superoxide generation ability and lignification ability was reported.

Localized and induced-systemic resistance against *Phytophthora parasitica* caused by the AM fungi, *Glomus mosseae*, has been observed in tomato roots (Cordier et al. 1998). The phenomena were demonstrated by use of a split-root experimental system. Decreased pathogen development in mycorrhizal and nonmycorrhizal parts of the root system was associated with an accumulation of phenolics and plant cell defense responses. *G. mosseae*-containing cortical cells in the mycorrhizal tissues were immune to the pathogen infection and exhibited a localized resistance response with the formation of cell wall appositions reinforced by callose adjacent to intercellular hyphae. The systemically induced resistance in nonmycorrhizal root parts was characterized by elicitation of host wall thickenings containing nonesterified pectins and PR-1a protein in reaction to the intercellular hyphae of the pathogen. Systemic resistance was also characterized by the formation of callose-rich encasement material around *P. parasitica* hyphae that were penetrating root cells and PR-1a protein was detected in the pathogen wall only in these tissues. None of these cell reactions were observed in nonmycorrhizal pathogen-infected root systems, where disease development resulted in host cell death. Increased chitinase activities have also been reported in AM symbiosis as part of the induced defense reaction by these mycorrhizal fungi. Pozo et al. (1999) studied β -1,3-glucanase in tomato roots which were either colonized by AM fungi and/or infected by the pathogen *Phytophthora parasitica*. β -1,3-glucanase activity was higher in mycorrhizal roots compared to the non-mycorrhizal roots. Nonmycorrhizal roots infected by *P. parasitica* showed high levels of activity but the pathogen did not induce β -1,3-glucanases in AM colonized roots. There was strong evidence to suggest that these hydrolases are antifungal proteins. Increased chitinase activities have also been reported in ectomycorrhizal symbiosis (Albrecht et al. 1994; Sauter and Hager 1989).

5 CONCLUSIONS

The original purpose of isolating beneficial microorganisms from soil, especially from the rhizosphere of plants, was to obtain microorganisms, which showed a growth promotion effect in plants. In addition to this effect, subsequent studies have investigated direct suppressive effects on pathogens. Due to the large amount of research undertaken over the years, we now know that this view is limited. This chapter has focused on PGPF, mycorrhizal fungi, and endophytic fungi, all of which show efforts as plant growth promoters and disease suppressors, and the mechanisms of mineralization, hormone production, antagonism, and induced resistance have been considered. These mechanisms are commonly involved in plant-growth promotion or disease suppression by PGPF, mycorrhizal fungi, and endophytic fungi to some degree depending on the fungi studied. These fungi are symbiotic to plant roots, and so they offer advantages for keeping the plant healthy for long durations. In addition, mineralization and hormone production by these fungi have the potential to substantially improve agricultural productivity and to reduce environmental costs. The reduction in nitrogen fertilizer usage due to the use of these fungi can be expected to substantially reduce nitrate pollution of ground and surface water. In addition to these antagonistic activities, induced resistance in plants treated with these fungi broadens the potential range of pathogens that may be controlled. This resistance coupled with long-term colonization should provide important new tools for highly economical pest control with minimal environmental pollution.

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Challenges and Strategies for Development of Mycoherbicides

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1 INTRODUCTION

The application of biological control for the management of weed populations has generally been viewed as an environmentally sound additional approach to chemical herbicides (Boyetchko et al. 2002; Mortensen 1998; Roskopf et al. 1999). Bioherbicides are often described as the intentional use of plant pathogens that are mass-produced, formulated, and applied at high inoculum rates in a similar fashion as chemicals. Although a variety of microbial agents may be used, host-specific fungal pathogens often referred to as mycoherbicides, have been studied more extensively for biocontrol of weeds. In comparison, classical biological control involves the importation of natural enemies and relies on the natural survival, dissemination, and self-perpetuation of the living agent for control of weeds below ecological thresholds. The classical approach is often considered more appropriate for low management systems such as pasture and rangeland where site disturbance is minimal, while bioherbicides are ideal for single-season management of agricultural and forest weeds where site disturbance is the norm. Despite many economic, social, and environmental benefits ascribed to biological control, it is reasonable to ask why more bioherbicide or mycoherbicide products are yet to become widely available in the marketplace. Many researchers would argue that there has been a great deal of progress, with several additional microbial agents identified as potential bioherbicides and innovative improvements in mass-production, formulation, and application of living organisms. Despite the various accomplishments by researchers worldwide (Boyetchko et al. 2002; Charudattan 2001), the question remains whether we have made significant advancements in bioherbicide research that would facilitate increased adoption of this technology. Several reviews have discussed in great

detail some of the limitations associated with bioherbicides (Auld and Morin 1995; Makowski 1997; Mortensen 1998), including biological, environmental, and technological constraints. Critics have attributed these constraints to the lack of further development of many biocontrol agents. This review will provide an update on the status of mycoherbicide research, summarize some of the challenges encountered, and provide some thoughts on potential new approaches that may be used to address these challenges in order to advance the development of promising mycoherbicide candidates.

2 STATUS OF BIOHERBICIDES

Several recent reviews have provided an overview on various bioherbicide projects being conducted around the globe (Boyetchko 1999; Boyetchko et al. 2002; Charudattan 2001; Roskopf et al. 1999). Eight bioherbicides have been registered in various countries over the last two decades with several other microbial candidates in various stages of evaluation and development (Table 1). Devine[®] and Collego[™], the first mycoherbicides registered in the United States are currently marketed by Encore Technologies (Minnetonka, MN), while Stumpout[®], a wood-decaying fungus used to control resprouting of *Acacia* spp., is commercially available in South Africa. *Chondrostereum purpureum* is a wound pathogen that reduces regrowth of competing hardwood tree species and is marketed as BioChon[™] by Koppert Biological Systems in the Netherlands. A Canadian strain of the pathogen is also currently undergoing registration approval through the Canadian Pest Management Regulatory Agency and U.S. EPA and will be sold as Chontrol[®] by MycoLogic (W.E. Hintz, MycoLogic, Inc., personal communication). Another mycoherbicide

Table 1 Examples of mycoherbicide agents at various stages of development and commercialization

Status	Pathogen (Trade Name ® or ™)	Target weed	Country
Commercially available	<i>C. gloeosporioides</i> f. sp. <i>aeschynomene</i> (Collego®)	Northern Jointvetch	USA
	<i>P. palmivora</i> (Devine®)	Stranglervine	USA
	<i>C. laeve</i> (Stumpout®)	Black & golden wattle	South Africa
	<i>C. purpureum</i> (BioChon™)	Hardwood tree species	Netherlands
Registered, not commercially available	<i>C. gloeosporioides</i> f. sp. <i>malvae</i> (Mallet WP) ^a	Round-leaved mallow	Canada, USA
	<i>P. canaliculata</i> (Dr. Biosedge®)	Nutsedges	USA
Precommercial development	<i>C. purpureum</i> (Chontrol®)	Hardwood tree species	Canada, USA
	<i>A. destruens</i> (Smolder®)	Dodder	USA

^a Originally registered in Canada as BioMal® by Philom Bios; licensed to Encore Technology for registration as Mallet WP in Canada and the United States. Mycoherbicide not being further developed due to technical difficulties in mass production.

currently undergoing review is *Alternaria destruens*, under the name Smolder®, for control of dodder. Other examples of mycoherbicides have been discussed in greater detail by Boyetchko (1999), Boyetchko et al. (2002), and Roskopf et al. (1999). Charudattan (2001) has also compiled a comprehensive list of bioherbicide projects worldwide. While the number of commercial bioherbicides appears to be limited, Charudattan (1991) calculated a success ratio of 20:1 for bioherbicide development compared to the success rate of less than 1% for chemical herbicide compounds evaluated and developed by chemical companies. This success ratio begins to look even more encouraging for bioherbicides when developmental costs are taken into consideration. Charudattan (2001) estimated that the resources and capital required for a chemical company to conduct research and development and register chemical herbicides is approximately US\$ 50 million in comparison to US\$2 million for bioherbicides. Nevertheless, a very small portion (i.e., less than 1%) of the commercially available weed control products are represented by bioherbicides and investment in these microbial-based products has largely been by small-to-medium sized enterprises. Government and university institutions have invested infrastructure and expertise in this area and it has been through such efforts that this technology has been transferred to industry.

3 CHALLENGES IN BIOLOGICAL CONTROL

Several reviews have provided a suggested list of desirable characteristics for a bioherbicide candidate in order for it to be successful (Charudattan 1991; Makowski 1997; Mortensen 1998). Generally, these traits include: (a) a narrow host range, (b) ease of use, (c) genetic stability, (d) ability to mass produce inoculum cost-effectively with long shelf life, and (e) ability to be fast-acting with predictable field performance and provide sufficient weed control comparable to chemical herbicides. Many of these traits, along with the term bioherbicide (mycoherbicide), may create unrealistic expectations that all bioherbicides should eradicate weed

populations, similar to chemical herbicides (Auld and Morin 1995). The challenges that have limited the advancement of bioherbicides have been categorized into four constraints: (a) biological, (b) environmental, (c) technological, and (d) commercial. While the commercial consideration is important, this review will focus on addressing the other three constraints. Researchers can make pragmatic decisions on the selection of an appropriate target weed that may have impact on the market decisions by industry to invest in the development of bioherbicide agents, but the regulatory environment for registration of such products is often affected by political will and/or policy of individual governments.

3.1 Biological Factors

Weeds are inherently variable by nature, with many weed species possessing several biotypes. For this reason, the genetic diversity of weed populations can present several challenges when researchers evaluate specific isolates or strains of fungal pathogens (Auld and Morin 1995; Boyetchko et al. 2002). Such is the case with yellow nutsedge that was found to have extensive genetic variability within and between populations and, therefore, greater variability in susceptibility to the bioherbicide agent *Puccinia canaliculata*, compared to purple nutsedge populations (Horak et al. 1987; Okoli et al. 1997). The success of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* as the mycoherbicide Collego® was attributed to the uniform susceptibility of the weed northern jointvetch (Templeton et al. 1984). A better understanding of the target weed population structure will contribute to the effective search for bioherbicide agents with consistent performance on genetically diverse weed species (Boyetchko et al. 2002). Another consideration is the view that all bioherbicide candidates must be specific to one particular weed species. The advantage of a highly host-specific bioherbicide candidate is the assurance that nontarget, beneficial plant species, or crops closely related to the weed will not be affected (Makowski 1997; Mortensen 1998). However, a strict host-range requirement may not be

economically feasible because the majority of agroecosystems are comprised of multispecies weed communities (Frantzen et al. 2001). Plurivorous pathogens may be used safely under certain circumstances when they can be separated sufficiently from nontarget hosts in space and time (De Jong et al. 1999). Plant architecture and morphology have played a role in the success or failure of bioherbicide agents. The majority of weed species selected as targets for biological control have been broad-leaved weeds using foliar fungal pathogens (Charudattan 1991; 2001). Grasses are considered more difficult to control because the meristem is covered by a leaf sheath thereby prohibiting direct attack by the pathogen (Greaves and MacQueen 1992). Grass weeds are also closely related to many crops (e.g., cereals) in which they occur, making selectivity of mycoherbicide agents more challenging (Wapshere 1990). A particularly important factor with perennial weeds is their regeneration via rhizomes and stolons, which makes long-term weed control difficult (Greaves and MacQueen 1992). This often results in the weed out-growing the disease caused by foliar-applied bioherbicide candidates. However, there are many prospective soilborne fungal and bacterial agents that may be used as pre-emergent bioherbicides to control Poaceae and perennial weed species (Boyetchko et al. 2002). Other physical barriers such as leaf hairs and waxy cuticle layers may act as impediments to infection and establishment of fungal pathogens on the phyllosphere (Auld and Morin 1995). Although high inoculum applications have been used to overcome biological constraints resulting from low infection efficiency or virulence of the pathogen, these rates may not be technologically feasible due to plugging of spray equipment or economically viable from a production standpoint.

3.2 Environmental Factors

Two major limiting factors that have an impact on mycoherbicides are temperature and moisture requirements (Auld and Morin 1995; Makowski 1997; Mortensen 1998). TeBeest et al. (1992) considered temperature to be less important than moisture in most cases because many fungal pathogens will infect plants over a broad range of temperatures. However, there is often an interaction between temperature and moisture that has a greater effect than temperature alone. Free moisture and leaf wetness duration can significantly affect the ability of the fungal pathogen to germinate, produce penetration structures, and ultimately cause plant infection. This requirement of leaf wetness duration often increases when temperatures are in suboptimal ranges. Early evaluations of potential mycoherbicide candidates have often been conducted where dew periods in excess of 12 h are provided to ensure a high rate of infection on the weed (Lawrie et al. 1999; McRae and Auld 1988; Morin et al. 1990a). Green and Bailey (2000a,b) reported that *A. cirsinoxia* for control of Canada thistle required at least 8 h

of continuous leaf wetness. The pathogen infected the weed under a broad range of temperatures (10–30°C) when free water or high relative humidity was present, but long durations of intermittent leaf wetness was detrimental to survival of germlings and, therefore, not conducive to high bioherbicidal activity. Some pathogens such as rust fungi are wind-dispersed and generally require less moisture than water-disseminated pathogens such as *Colletotrichum* spp. where spores are contained in a mucilaginous matrix (Hasan and Wapshere 1973; TeBeest 1991). Makowski (1997) further reiterated that evaluating the impact of environmental parameters under controlled conditions may provide clues about potential performance of bioherbicide candidates, but further investigations under variable field conditions where these factors are difficult to control are more complicated.

3.3 Technological Factors

The feasibility of commercializing bioherbicide agents has often been dependent on the ease and economics of mass-producing and formulating large amounts of viable, stable, and highly efficacious microbial propagules (Auld and Morin 1995; Mortensen 1998; Slininger et al. 1998). It is generally believed that submerged liquid fermentation is the most efficient commercial mass-production method for most biocontrol agents (Jackson et al. 1996). Although general guidelines or common fermentation ingredients for medium composition are available in public domains (Stanbury et al. 1995), most commercial fermentation protocols are custom designed for a specific organism and details are generally treated as trade secrets. However, when a company licenses a bioherbicide technology, the ability to mass-produce the agent economically and market it at a cost that is affordable to farmers, represents a strong determining factor in its development and commercialization potential. Lack of reliable field performance due to inadequate formulation and application technology has often been cited as a major reason for the lack of progress in bioherbicides (Greaves et al. 2000; Peng et al. 2001). It has been claimed that suitable formulation technology may help address some of the environmental constraints, particularly moisture requirements that often hinder the advancement of bioherbicide candidates beyond the discovery and evaluation phase. Propagules of foliar-applied fungal agents generally require free water to germinate and penetrate weeds. This leaf wetness requirement and its interaction with the ambient temperature often determine the outcome of a mycoherbicide application (Zhang and Watson 1997). Often, researchers evaluate mycoherbicide candidates by spraying till runoff, thereby overestimating the potential of many bioherbicide candidates at the early stage of evaluation (Lawrie et al. 1999). Less stringent dew requirement may be an advantage for foliar agents, especially in semiarid climates such as the Canadian prairies, where rainfall is infrequent at critical periods (e.g., in the spring). Intermittent dew occurs more often than

continuous dew, but germinated fungal spores under short dew periods are more sensitive to desiccation and UV irradiation (Green and Bailey 2000b), therefore, survival of the germings is going to be the key to successful infection under intermittent dew.

4 APPROACHES FOR OVERCOMING CONSTRAINTS

Most mycoherbicide programs are initiated through surveys to discover fungal pathogens exhibiting bioherbicidal potential, followed by a series of biological and ecological assessments to determine the feasibility of mycoherbicide candidates. However, a pragmatic approach of selecting appropriate mycoherbicide candidates is required and should be considered as a continuum amongst several factors that will ultimately influence the field performance of the fungal pathogen (Figure 1). While nutritional and physical factors are vital during fermentation, down-stream processing is equally important for an efficient mass-production system. Selection of appropriate formulation technology is influenced by fermentation processes and should be based on critical limitations such as shelf life and environmental constraints encountered with mycoherbicide development. Formulation ingredients can affect delivery and application of the mycoherbicide agent. If these ingredients result in the inability to deliver the fungal pathogen to the target weed (e.g., high viscosity and ultimate plugging of equipment), effective weed control will not be achieved. Often, mass-production, formulation, and application can be interrelated, therefore, changes in one of

these processes may impact the others. At any stage of evaluation, refinements or modifications to these processes, even minor, may result in significant improvements in mycoherbicide performance.

4.1 Selection and Improvement of Bioherbicide Agents

On average, chemical companies screen more than 60,000 compounds before a new active ingredient of pesticide can be determined. The number required for screening biocontrol agents should be less due to a relatively smaller range of variations amongst naturally occurring fungal populations. However, if we are to identify “nature’s best,” a systematic approach is essential during the exploration and discovery phase to thoroughly evaluate the biodiversity. This diversity provides excellent opportunities for finding fungal strains with potential suitable traits for biocontrol (Avis et al. 2001; Weidemann and TeBeest 1990). Substantial variations may exist amongst different strains of a fungal species in terms of its virulence and responses to environmental variables (Sands et al. 1997; Tessmann et al. 2001). To be effective, critical traits for selection should be clearly identified and sensitive bioassays developed. Pathogen strains with high levels of virulence may exist in nature at low frequencies due to higher extinction rates (Yang and TeBeest 1992). Results by Yang and TeBeest (1992; 1993) indicated that pathogens showing high virulence along with important epidemiological traits such as rapid infection rates and dispersal are more likely to be candidates of a successful mycoherbicide agent. The success

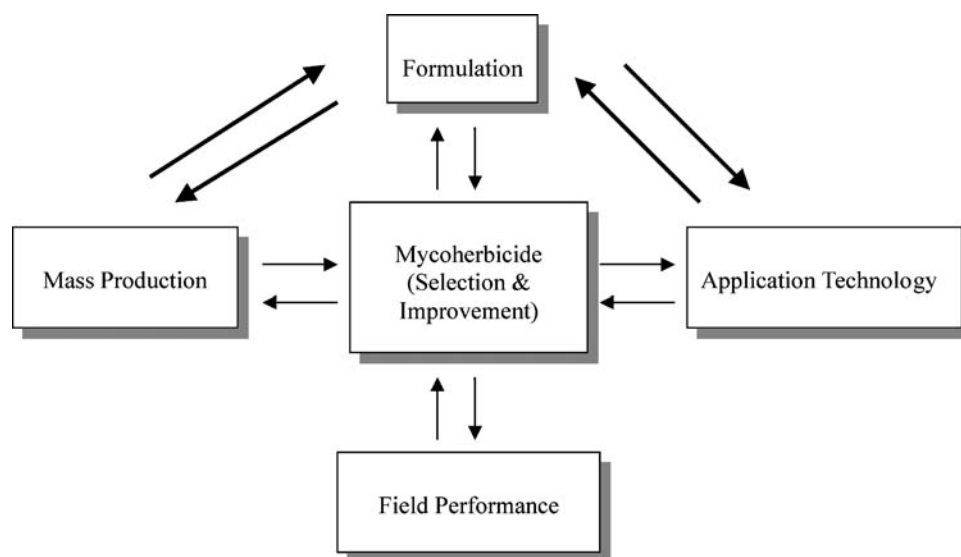


Figure 1 Strategic framework for evaluation and development of mycoherbicides.

of *C. gloeosporioides* f. sp. *aeschynomene* for control of northern jointvetch was partially attributed to its ability to easily spread as an endemic pathogen. Yang and TeBeest (1995) further demonstrated a rapid rate of mortality of the weed as the number of pathogen lesions per plant increased from a single lesion. Therefore, more aggressive and virulent isolates of a pathogen with high infection efficiency, shorter latent periods, and better sporulation from diseased tissues should be selected from amongst the pathogen population. Chemical and physical methods have been used to create fungal mutants with acquired new traits such as elevated antibiotic production (Graeme-Cook and Faull 1991) or increased biocontrol efficacy (Palani and Lalithakumari 1999). Stability or low reversion frequency was observed with some mutants but, in general, stability can be a concern with chemical and physical mutagenesis (Wibowo et al. 1999). Ziogas et al. (1995) reported UV-induced mutants of *Nectria haematococca* with variable tolerance to fungicides that showed the same level of fitness as wild types as expressed by the rate of growth and virulence on squash seedlings. Mutagenesis is apparently a quick way of creating new fungal strains with variable traits. However, efficient bioassay systems based on the understanding of critical constraints are needed for an effective selection strategy. It is not uncommon for induced mutants to have lower competitiveness than the wild type due to reduced infectivity or reproductivity (Yang and TeBeest 1995), but judicious use of this technique may help develop new mycoherbicide strains that overcome critical hurdles such as those demonstrated with the plurivorous pathogen *Sclerotinia sclerotiorum* (Miller et al. 1989).

4.2 Fermentation/Mass Production

There are potentially three fermentation systems that may be used for mass production of mycoherbicide agents: submerged liquid culture, solid substrate fermentation, and two-phase system (Auld 1993a). At the industrial level, liquid fermentation is the most common method for economical production of microbial inoculum. Two commercial mycoherbicides, Collego® and Devine®, are manufactured this way in the United States (Churchill 1982; Stowell 1991). By understanding the importance of nutritional and environmental factors on induction of fungal sporulation, spore yield, and bioherbicidal efficacy, rational approaches can be taken to develop the most efficient liquid production procedures for mycoherbicide agents. Jackson and Bothast (1990) reported that carbon concentrations and C/N ratios are the key to sporulation of the mycoherbicide agent *C. truncatum*. Carbon concentrations ranging from 0.4 to 1.5% gave highest spore yields while higher concentrations (2–4%) inhibited sporulation. Similarly, a C/N ratio at 15:1 was more favorable for sporulation than 40:1 or 5:1. In a further study, it was revealed that nutritional aspects impacted not only spore yield, but also spore efficacy of *C. truncatum* in controlling hemp sesbania (Jackson et al. 1996). Morin et al. (1990b) also observed that

sporulation of *Phomopsis convolvulus* was completely inhibited when the C/N ratio was reduced from 1:1 to 1:5 using modified Richards medium, but the effect on spore efficacy was not reported. By understanding these impacts, fermentation procedures can be fine-tuned to maximize the production and potency of mycoherbicide agents. One of the drawbacks with liquid fermentation is that down-stream processing can be more complicated and costly. Large centrifuges are normally required to spin off spores and often a large number of spores can be imbedded in the mycelial biomass (Auld 1993a). Following recovery from the fermentor, it is usually necessary to dry the spores for long-term storage but retaining spore viability during the drying process may not be easy. Generally, spores should be dried rapidly and gently, but the drying conditions will vary with each organism. Drying methods frequently used include freeze, air, spray, or fluid-bed drying, or a combination of these methods (Churchill 1982). Solid substrate fermentation is used less commonly in commercial production of microbes except for the mushroom spawn industry. Often defined nutrients are added with liquids or solid materials such as vermiculite or paper pellets (Auld 1993a). Various cereal grains have been used to produce fungal inoculum and it is relatively easy to quantify and disperse the inoculum on these solid substrates (Boyette et al. 1991). In some places nutritive solid substances such as nutshells or straw may be available locally at low cost. Higher labor costs, difficulties in maintaining sterility, lack of control of cultural conditions, and recovery of spores from the substrate are inherent problems with solid substrate fermentation (Churchill 1982). Pfirter et al. (1999) evaluated a variety of solid substrates and found that *Stagonospora convolvuli*, for control of field bindweed, sporulated the best on cous-cous (cracked hard wheat) followed by maize semolina, yielding 5×10^8 spores/g substrate and 3×10^8 spores/g, respectively. Morin et al. (1990b) also reported the production of 7×10^8 conidia/g with *P. convolvulus* using pot barley grain as a solid substrate. They also compared liquid and solid fermentation methods and found that conidia produced using both systems were morphologically similar and there were no differences in pathogenicity. Particle size, moisture content, and temperature appear critical for successful solid substrate production. A mycoharvester developed at CABI Bioscience (www.dropdata.net/mycoharvester) appears to be a simple device for collecting spores of the mycoinsecticide fungus, *Metarhizium anisopliae*, produced on rice grains. This device has also been attempted to reduce inoculum impurity of the mycoherbicide *Pyricularia setariae* (Gary Peng, unpublished data). By reducing the proportion of large particles in the inoculum, the mycoherbicide can be applied at high spore concentrations and low carrier volumes using common spray equipment (Peng et al. 2001). A two-phase system produces mycelium in deep tank fermentation followed by sporulation in shallow open trays. This method may be particularly useful for fungal agents that cannot be manipulated to sporulate in submerged culture, but this system is labor-intensive and expensive, and additional handling of the material may lead to

contamination of the final product (Roskopf et al. 1999). Liquid fermentation produces a large amount of biomass efficiently and sporulation in a “dry phase” may circumnavigate costly down-stream processing issues. Walker (1980) used *A. macrospora* as a model system to first produce fungal mycelium in a liquid, then homogenize and mix it with vermiculite, followed by thinly spreading the homogenate on a solid surface. Using a similar system, Walker and Riley (1982) successfully produced spores of *A. cassiae*, a mycoherbicide agent for control of sicklepod. One area of fermentation that can often be overlooked is the feasibility of scale-up from shake flask laboratory volumes to production-plant level. Optimization of fermentation conditions and media components can be readily achieved in the laboratory while pilot-plant scale fermentation can help to effectively verify these selected parameters (Kwanmin 1989). Some sophisticated pilot-scale devices with consistent designs to large-scale facilities are now available. These units are particularly useful for scale-up studies and provide a wide range of pH, agitation speeds, impeller designs, aeration rates, choices of regulated incoming gases, variations in baffling and background pressures, and temperatures (Churchill 1982). However, conditions required to reach optimal yield, costs, and efficiencies in production scale fermentation can be more difficult to achieve. Some biological factors that need to be considered are culture stability, number of generations, and mutation rate while chemical factors include pH, water quality, and fermentation medium quality. Physical factors that should be evaluated include aeration, agitation, pressure, temperature, and medium sterilization. Kwanmin (1989) indicated that factors that may not have been important at a smaller scale could have significant impact on the operation and design of fermentation processes at the production plant level.

4.3 Formulation

It is believed that many of the environmental challenges, particularly long dew period or leaf wetness requirements, can be tackled to a large extent with formulation technologies (Boyetchko et al. 1999; Greaves et al. 2000; Green et al. 1998). Formulation is essentially the blending of microbial propagules with a range of carriers or adjuvants to produce a form that can be effectively delivered to target weeds. For microbial agents, formulation may enhance pathogen survival and infection as well as extend propagule stability and product shelf life. Depending on the type of organism, mode of action, and available spray equipment, formulation ingredients vary substantially. For instance, foliar-applied agents may be exposed to rain-wash, UV irradiation, and desiccation prior to germination and penetration (Rhodes 1993). Therefore, various adjuvants with adhesive, sun-blocking, or humectant properties have been suggested to alleviate the negative impact by these factors (Schisler et al. 1995; Womack and Burge 1993). Formulations that increase moisture-retaining properties, reduce the rate of evaporation

and/or enhance the rate of infection of the mycoherbicide agent should be explored to address dew limitations. In the literature, emulsions and hydrophilic polymers are reported most frequently to improve the performance of foliar-applied mycoherbicide agents. Formulation research has focused particularly on desiccation and dew requirements of fungal agents during the infection process and incremental to drastic improvements have been seen in different studies (Auld 1993b; Connick et al. 1990; Lawrie et al. 2000; Shabana 1997). There is a growing belief that innovations in formulation will be a vital component to the success of the next generation of bioherbicides, especially for foliar-applied products (Greaves et al. 1998). For best results, formulations should predispose weeds to infection by pathogens and buffer pathogen propagules against environmental extremes while promoting disease development. Nutrient supplements, including simple sugars, amino acids, pectins, salts, and plant extracts have been added to formulations to stimulate the infection process and protect germinating propagules, but these nutritional effects are often agent specific (Bothast et al. 1993; Schisler et al. 1995; Womack and Burge 1993). Exogenous nutrients may stimulate germination and growth of many fungi, but frequently appressorial initiation is even more important to plant penetration and infection. Oversupply of nutrients can lead to excessive growth of germlings, delaying, or even reducing appressorial formation and penetration (Takano et al. 1997). Tremendous efforts have been made on developing various emulsions to alleviate moisture constraints, thereby enhancing field performance. Invert emulsions showed the most impressive results by reducing or even eliminating the need for dew with several fungal agents (Connick et al. 1991b; Yang et al. 1993). Bioherbicidal control of hemp sesbania using *C. truncatum* in an invert emulsion was significantly enhanced under field conditions (Boyette et al. 1993). Invert emulsions consist of water droplets suspended in oil and evaporation of the trapped water is dramatically reduced and microbial propagules held in the water are, therefore, protected (Daigle et al. 1990; Womack and Burge 1993). Despite the apparent improvement, invert emulsions can be very complex, difficult to apply using existing spray equipment due to extreme viscosity and may exhibit phytotoxicity in many plant species (Boyette 1994; Womack et al. 1996). Although these invert emulsions have been used to expand the host range of mycoherbicides (Yang and Jong 1995), this change may also affect nontarget crops. High oil content also makes invert emulsions more costly, especially when high spray volumes are required. Being nonevaporative, oils were considered a compatible carrier with ultra-low volume application techniques for the mycoinsecticide *M. anisopliae* under extremely dry conditions (Bateman and Alves 2000). In contrast to invert emulsions, oil suspension emulsions are considered to be more practical because they have significantly lower oil content and can be applied with most existing spray equipment (Green et al. 1998). Fungal propagules can be first suspended in oils, then mixed with a much larger volume of water containing an emulsifier to make stable emulsions

(Auld 1993b). Klein et al. (1995) used suspension emulsions of *C. orbiculare* made from two vegetable and mineral oils that were mixed and applied with water ranging in concentrations from 0.5 to 10% for control of *Xanthium spinosum* under field conditions. These formulations enhanced weed control in several field trials compared to water as a carrier sprayed at similar application volumes. Oils may have variable effects on propagule germination and performance of mycoherbicide agents. Paraffin oils were toxic to spores of *Ascochyta pteridis*, a mycoherbicide candidate for bracken, *Pteridium aquilinum* (Womack et al. 1996), while unrefined corn oil enhanced spore germination of *C. truncatum* in emulsions containing 10–50% of oil (Boyette 1994; Egley and Boyette 1995). The mechanisms by which the oil suspension emulsions enhance mycoherbicidal activity are not well understood. Emulsions may help maintain the stability and infectivity of fungal propagules prior to onset of dew (Green et al. 1998). Spray retention is likely enhanced, reducing the spore dose required for effective weed control. Moisture retention has also been demonstrated with hydrophylic polymers such as Kelgin[®] HV, MV, LV, Kelzan[®] xanthan gum, Gellan gum, N-Gel[™], Metamucil[®], and Evergreen[®]500 (Shabana et al. 1997). These polymers enhanced viability, germination, and efficacy of the mycoherbicide agents *A. cassiae* and *A. eichhorniae*. Humectants such as psyllium (e.g., Metamucil[®]) are known to have high moisture retention properties and reduce the rate of moisture loss (Greaves et al. 2000). Coformulation of the mycoherbicide agent, *A. caulina*, with the skinning agent polyvinyl alcohol and Metamucil[®] enhanced control of *Chenopodium album* under reduced dew conditions. For mycoherbicides applied to the soil, encapsulation of the fungi in solid matrices is more suitable than liquid formulations. Calcium alginate has been used to mix fungal spores with a variety of carriers such as kaolin clay, ground oatmeal, soy flour, and cornmeal (Boyette and Walker 1986; Walker and Connick 1983; Weidemann and Templeton 1988). Conidial production and field efficacy can be enhanced by amending the mixture with various nutrients (Daigle and Cotty 1992; Weidemann 1988). “Pesta” has also been used as a type of granular formulation where fungal propagules are entrapped in a wheat-gluten matrix consisting of semolina flour, kaolin, and fungal biomass (Connick et al. 1991a). Further development of this process has resulted in the formation of uniform granules using a twin-screw extruder and by controlling the moisture content through fluid-bed drying (Connick et al. 1998).

4.4 Application Technology

Delivery and retention of sufficient number of fungal propagules on weeds can be very challenging. Most initial studies on mycoherbicide efficacy spray the inoculum to the weed foliage till runoff using aerosol sprayers. This spray method generally applies excessive volumes (up to 3000 l/ha) that can maximize the retention and exaggerate the potential

of biocontrol agents (Greaves et al. 2000). Disregard for appropriate methods of application can contribute to poor or inconsistent field performance (Smith and Bouse 1981). In most field crops, application volumes over 600 l/ha are considered high (Matthews 1992), and the trend is generally toward lower volumes.

Use of high spore concentrations can potentially reduce application volumes without compromising the efficacy of weed control (Peng et al. 2001). Increased propagule number may help improve the efficiency of foliar coverage by reducing the proportion of “empty” droplets (Jones 1998), but also pose high requirements on quantity and quality of the inoculum necessary to achieve the desired level of weed control. Mycelium clumps or other impurities in extremely concentrated formulations can also easily plug up the spray system. Inoculum concentration, carrier volume, and other spray parameters need to be studied jointly to optimize spray results (Jones and Burges 1998). Nordbo et al. (1993) suggested use of fast travel speed to improve spray retention on vertical leaf surfaces. In a recent study, Peng et al. (2001) observed that a finer droplet spectrum combined with more horizontal trajectories enhanced retention efficiency on green foxtail. Richardson (1987) and Spillman (1984) made similar suggestions on application of herbicides. These retention characteristics have also been discussed by Jones (1998) and Reichard (1988), and have been used to explain improved spray results in a number of herbicide studies (Knoche 1994). It needs to be recognized that there are limitations with manipulation of certain spray parameters. For instance, too fine a spray may not be practical in every case depending on the size of mycoherbicide propagules and due to potential spray drift concerns (Jones 1998). Interpretation of droplet size spectra for optimal dose transfer of biopesticides is required, and this will vary with the mycoherbicide agent, particularly in relation to the spore size and morphology (Bateman 1999). Although fine tuning of application parameters can improve spray characteristics, it is more important to determine if these improvements can be translated into meaningful enhancements of weed control efficacy.

4.5 Herbicide Synergy

As stand-alone products, mycoherbicides have achieved limited success in the marketplace. Technologies such as formulation and application methods can improve the performance of biocontrol agents, but more noticeable enhancements in weed control have been observed with the combined application of mycoherbicide agents and synergistic herbicides (Peng et al. 2000; Sharon et al. 1992; Wymore et al. 1987). Limited information seems to suggest that synergistic interactions are herbicide and pathogen specific. Use of glyphosate on Canada thistle assisted weed control by *A. cirsiinolia* only marginally, especially under field conditions (Bailey et al. 2000). Peng et al. (2000) compared two groups of herbicides, bentazon and metribuzin, for

interaction with a fungal pathogen on scentless chamomile, a noxious weed in the Canadian prairies. Preliminary results revealed significant fresh weight reduction by up to 150% with applications of herbicides plus the pathogen compared to herbicides alone. Tank-mixing of *C. coccodes* with thidazuron (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-yl-urea) also increased the mortality of velvetleaf when compared to the application of the mycoherbicide alone in field trials (Wymore et al. 1987). The application of a sublethal dose of glyphosate with *A. cassiae* resulted in an increase in susceptibility of the weed sicklepod (Sharon et al. 1992). As a result, equivalent control was achieved with five times less inoculum of the mycoherbicide agent. The herbicide was believed to interfere with the shikimate acid pathway that is involved in the elicitation of phytoalexins, low molecular weight antimicrobial compounds involved in a plant's defense response. Subsequent interference with the plant's defense mechanism resulted in greater susceptibility of the target weed to the mycoherbicide (Hoagland 1996). This synergistic interaction appears to be an attractive mechanism to enhance the effectivity and feasibility of mycoherbicide agents. According to Hoagland (1996), several benefits may be captured with the application of microbe/herbicide synergy: (a) when defense capabilities of weeds are lowered using herbicides, weeds become more susceptible to pathogen attack, (b) the quantity of mycoherbicide agent or the application rate of herbicides may be reduced, and (c) host range of a given mycoherbicide agent may be expanded with the use of selected chemical synergists.

5 CONCLUSION

The identification of efficacious biological control agents is only the beginning in the development of mycoherbicide products. Continuing strain selection is essential to ensure that "nature's best" is employed. Likely there will always be limitations associated with naturally occurring organisms, therefore, enabling technologies such as fermentation, formulation, and application technology will be instrumental in determining whether a highly efficacious agent can be developed into an economically feasible mycoherbicide product. The ultimate goal is to incorporate mycoherbicides into agricultural production systems. More efforts should be directed into combining biocontrol agents with other weed control options including chemical herbicides, cultural practices, and use of the multiple pathogens to enhance the effectiveness and flexibility of integrated weed management systems and to reduce the chemical load on crops and in the environment (Boyetchko et al. 2002; Roskopf et al. 1999). The challenge is to critically evaluate the merits of individual mycoherbicide candidates and to make realistic decisions whether they have all the essential characteristics required for successful application in agroecosystems. Based on discussions presented in this review, the following research priorities are suggested for development of mycoherbicide candidates:

- (a) Assessment of natural strain variation and biodiversity within the pathogen population based on critical epidemiological characteristics, including environmental adaptation, virulence, dispersal, and infection efficiency.
- (b) Evaluation of key areas for efficient scale-up mass-production based on fundamental elements relating to nutritional and physical requirements of specific fungal agents that facilitate selection of economical fermentation ingredient substitutes, along with down-stream processing procedures that are compatible with production methods.
- (c) Selection of appropriate formulation technologies (i.e., liquid or solid-matrices) based on the mode of attack and critical efficiencies of candidate agents. Over-simplification of formulation by using single ingredients will not likely address the complex challenges that fungal organisms will encounter in the environment, including moisture constraints, temperature extremes, and UV irradiation.
- (d) Critical factors in application technology related to placement and penetration of the crop canopy to the target weed in order to maximize application efficiency. These factors include leaf-wetting properties and ability to penetrate physical barriers (e.g., waxy cuticles and leaf hairs), retention and dispersal on the leaf surface, optimum dose transfer in various liquid droplets or solid-based granules, and selection of application equipment such as nozzle types and angle position or soil-application placement (e.g., within furrow application, side-banding, etc.). Application parameters should also be evaluated jointly with formulation ingredients.
- (e) Integration of mycoherbicide agents into crop production systems using several weed management tools (e.g., synergy with chemical herbicides, combinations with other biological control agents, or weed control options) to optimize weed control effectiveness. Judicious use of mycoherbicides as one of the components in an integrated weed management system will enhance their value and practicality for control of multispecies weed communities in agroecosystems.

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Biofungicides

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1 INTRODUCTION

At present, approximately 200 different fungicides have been introduced into agriculture and horticulture worldwide. Despite the enormous advances in chemical management of fungal diseases, some of the important plant pathogens such as vascular wilt, anthracnoses, take-all of wheat, and other root infections remain uncontrolled by current fungicidal chemicals (Knight et al. 1997). The build-up of resistant strains of target pathogens and the increasing public concern about synthetic fungicides have intensified the need for better and safer compounds in terms of novel modes of action, low rates of use, and low toxicological and environmental risk (Godfrey 1994; Tanaka and Omura 1993). As the environmental and commercial requirements for new fungicides become more demanding, it is increasingly difficult to discover new class of compounds to justify the effort and the costs of development. In order to get a chance to discover new fungicides that meet the mentioned characteristics, the exploitation of biologically active natural products is becoming mainstream in antifungal agent research.

Microbial metabolites represented by antibiotics have a number of chemical and biological merits as fungicides. Microorganisms are capable of synthesizing versatile chemical structures with diverse biological activities beyond the scope of synthetic organic chemistry (Porter 1985). An unexpected and newly found chemical structure is more likely to have new fungicidal activity and mode of action, especially showing no cross-resistance to the commercial fungicides (Früh et al. 1996). Biodegradability is the next property of microbial metabolites that cannot be overlooked. They degrade usually within a month or even a few days, when

exposed to agricultural environment, thus leading to the low residual level less harmful to the natural ecosystem (Tanaka and Omura 1993).

Microbial metabolites can be exploited in a number of different ways for the development of new fungicides. They can be directly used as fungicide products or as leads for the design of novel synthetic products. Alternatively, they can be used to highlight novel mode of action available as a new screening target. The recent successes in fungicide development came mainly from the discovery of potent lead compounds followed by chemical modifications that gave additional useful features fungicides. Potent antifungal activity is not the only factor to decide whether the microbial metabolite can be used as a commercial fungicide. Along with the chemical stability in the field, it should also have residual activity enough to reduce the application time to the economical level and low volatility sufficient to stay on the surface of host plants. Overall, it is very unlikely that a newly discovered microbial metabolite might possess all of the desired properties. Therefore, moving away from the viewpoint of antifungal metabolites as final products, microbial metabolites are currently reexploited as a source of enormously diverse chemical library that can supply lead compounds for development of fungicides. As seen in the example of the successful development of methoxyacrylates that are expected to be a major fungicide class in the future, it is not surprising that natural products are facing a revival as lead compounds for fungicide development.

The advances in molecular, biological, and chemical techniques made it possible to reinvestigate microbial metabolites from a totally different point of view. The increasing knowledge about the complex multidisciplinary

mystery of antifungal activity enables us to design a rationalized screening system based on the mode of action. Along with the innovative screening systems, the powerful instruments available for purification and structural elucidation of natural products have made it possible to adopt a high throughput approach to natural product screening (Bindseil et al. 2001).

In this chapter, we will review (a) microbial metabolites currently used as fungicides, (b) on-going efforts to discover lead compounds from diverse microbial sources, and (c) fungal specific targets to be used for screening of potential antifungal leads. In the later part of this review, we will discuss (c) trends in biofungicide research and interdisciplinary approaches to diversify their chemical library, which may yield novel antifungal compounds in the future.

2 MICROBIAL METABOLITES IN USE AS BIOFUNGICIDES

The most important antifungal metabolites in commercial use are listed below, which are applied to control fungal diseases on rice, vegetable, and fruits. The relative importance of the microbial compounds, when compared to synthetic fungicides, might have been undervalued because of several reasons such as the limitation in their spectrum of activity and in certain instances, the development of resistance. Nevertheless, the excellent activity of these biofungicides inspired to launch the screening programs for antifungal microbial metabolites, which resulted in profound chemical libraries of natural products (Godfrey 1994; Knight et al. 1997).

Blasticidin S, the first microbial fungicide available for plant protection, has been used practically for the control of rice blast disease caused by *Magnaporthe grisea*. Blasticidin S is a nucleoside antibiotic discovered from metabolites of *Streptomyces griseochromogenes* (Takeuchi et al. 1957). It potently inhibits the mycelial growth and conidial germination of *M. grisea*. The successful use of the compound encouraged further screening of microbial fungicides that eventually brought out kasugamycin, polyoxin, validamycin, and mildiomyacin.

Kasugamycin is an amino-sugar compound discovered in the metabolites of *Streptomyces kasugaensis* and *Streptomyces kasugaspinus* (Umezawa et al. 1965). It has *in vitro* antimicrobial activity against yeast and some plant pathogenic fungi including *M. grisea*. *In vivo* data showed that kasugamycin efficiently suppressed the development of *M. grisea* mycelia on rice plants by both preventive and curative treatments. However, it did not appear to inhibit the spore germination.

Polyoxins were isolated from the culture broth of *Streptomyces cacaoi* var. *asoensis* (Suzuki et al. 1965). The excellent *in vitro* activity and *in vivo* efficacy led to its commercial use for the control of fungal diseases of fruit trees and vegetables such as black spot of Japanese pear caused by

Alternaria kikuchiana and gray mold diseases caused by *Botrytis cinerea* (Isono et al. 1965).

Validamycin A produced by *Streptomyces hygroscopicus* var. *limoneus* was effective in controlling rice sheath blight caused by *Rhizoctonia solani* (Iwasa et al. 1970). Validamycin A was found to be a pro-drug, which is converted within the fungal cell to validoxylamine A, an extremely strong inhibitor of trehalase. This mode of action gives validamycin A a favorable biological selectivity, because the hydrolysis of the disaccharide trehalose does not occur in the vertebrates. The structural elucidation and total synthesis of validamycin A were achieved by Ogawa and coworkers (Suami et al. 1980).

Mildiomyacin is an aminoacylated nucleoside produced by *Streptomyces rimofaciens* (Harada and Kishi 1978). It was discovered by the method established to assay the control efficacy of antifungal agents against powdery mildew. Mildiomyacin has been known to act as an inhibitor of the fungal protein biosynthesis. Its low toxicity on vertebrates allows it to be an environmentally favorable crop protection agent.

3 MICROBIAL METABOLITES AS ANTIFUNGAL LEADS

The merits of natural products as fungicides can be a disadvantage in some respects. Their specific activity often resulted in a narrow antifungal spectrum with a limited application and the development of resistance strains under high selection pressure. Their biodegradability can also make them fragile, which results in short residual activity under harsh field conditions. These might be the reasons why the microbial metabolites used as commercial fungicide per se still commands less than 1% of total fungicide market (Tanaka and Ōmura 1993). Recently, a breakthrough in biofungicide research came from the semisynthetic approach using microbial metabolites as lead compounds. In particular, a far more promising and effective strategy for the development of new biofungicides is to use knowledge of the structure of antifungal compounds as the starting point for the synthesis of the compounds with optimized physical, biological, and environmental properties. The activity of natural products can in principle be improved by chemical modification. However, this approach relies heavily on the ready availability of sufficient quantities of the natural starting materials and the development of appropriate synthetic methodology. The biofungicides that were developed in this way are fenpiclonil and fludioxonil (Nyfeler and Ackermann 1992) and synthetic derivatives of antibiotic strobilurins such as β -methoxyacrylate azoxystrobin and kresoxim-methyl (Anke et al. 1977; Godfrey 1994). Such a derivative synthesized from microbial metabolites not only enhanced control efficacy but also improved properties such as photochemical stability, low cytotoxicity, and phytotoxicity. These successes encouraged the fungicide

researchers to search versatile lead compounds from diverse microbial sources with novel mode of action.

3.1 Recent Success in Fungicide Development from Antifungal Leads

Since strobilurin A and oudemansin A were found to be fungicidal metabolites in Basidiomycete fungi *Strobilurus tenacellus* (Anke et al. 1977) and *Oudemansiella mucida* (Musilek et al. 1969), respectively, a number of structurally related compounds were reported to have fungicidal activity. Each member of this family incorporates a methyl β -methoxyacrylate group linked at its α -position to a phenylpentadienyl unit, and all the compounds except strobilurin A carry either one or two additional substituents on the benzene ring that render structural complexity (Figure 1). Their mode of action on mitochondrial respiration, binding at a specific site on cytochrome *b*, is not shared by any other known class of fungicides (Sauter et al. 1995). The unique mode of action may not provide a chance of cross-resistance between β -methoxyacrylates and other fungicides. Although strobilurin A has excellent *in vitro* activity against a range of fungi, it did not show any useful *in vivo* activity in the greenhouse. This was due to its photochemical instability and relatively high vapor pressure, which cause it to disappear rapidly from a leaf surface. Through a series of synthetic program to solve these problems, azoxystrobin (Godwin et al. 1992) and kresoxim-methyl (Ammermann et al. 1992) were

developed as commercial fungicides that overcame the problems of the lead compounds (Clough et al. 1995). Azoxystrobin has a methyl β -methoxyacrylate toxophore, like strobilurin A, whereas kresoxim-methyl has a methyl methoxyiminoacetamide structure (Figure 1). Azoxystrobin has a wide antifungal spectrum against all four taxonomic groups of fungi and strong preventative activity, including inhibition of fungal germination (Heaney and Knight 1994). Kresoxim-methyl is also a broad-spectrum fungicide with strong antifungal activity against powdery mildew and apple scab (Brunelli et al. 1996). Considering its novel mode of action and amenability for synthetic approach, strobilurins are expected to be a major fungicide in near future.

Pyrrolnitrin is another example of a microbial metabolite used as a lead compound. Pyrrolnitrin, a secondary metabolite of *Pseudomonas pyrocinia*, which has a very simple structure, is thought to play a significant role in biocontrol activity of the bacterium (Arima et al. 1964). Although it showed excellent *in vitro* and *in vivo* activity in the greenhouse against *B. cinerea* and *M. grisea*, the disease-control efficacy in the fields was poor, because it rapidly decomposed when exposed to sunlight. In the extensive synthetic programs using pyrrolnitrin as a template, fenpiclonil (Nevill et al. 1988) and fludioxonil (Gehmann et al. 1990) were developed as seed-dressing agents against numerous fungal pathogens. The replacement of the chloro substituent in the 3-position of the pyrrole by a cyano group led to a remarkable enhancement in stability (Figure 1). Its biological activity also was optimized by appropriate substitution on the phenyl ring. Their improved photostability over pyrrolnitrin conferred the possibility as a foliar fungicide active against *B. cinerea*, *Monilinia* spp. and *Sclerotinia* spp. (Nyfeler and Ackermann 1992).

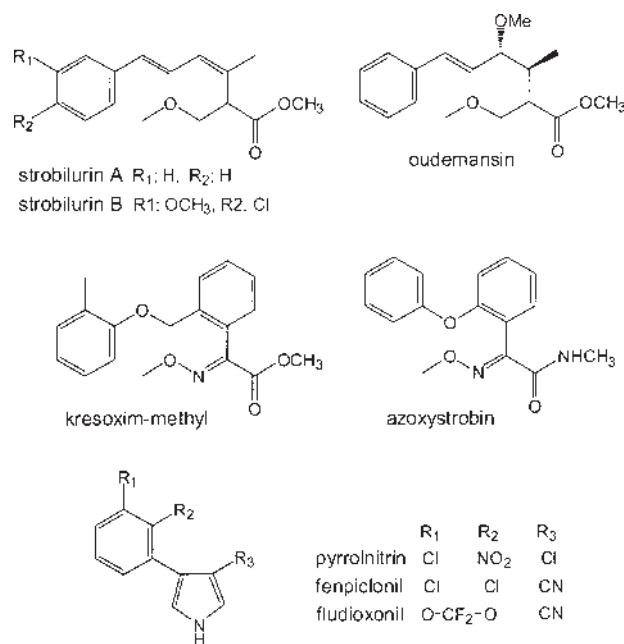


Figure 1 Chemical structures of biofungicides in practical use and their lead compounds.

3.2 Screening of Potential Leads from Diverse Microbial Sources

3.2.1 Streptomyces, the Largest Reservoir of Diverse Chemical Structures

Actinomycetes have been a major supplier of natural products (Huck et al. 1991; Lee and Hwang 2003). In particular, *Streptomyces* is a prolific producer of versatile structures of antibiotics. Most of antibiotics developed for agricultural uses including pesticides were isolated from *Streptomyces* strains (Tanaka and Ōmura 1993). Among antifungal antibiotics recently discovered from *Streptomyces* spp., polyketide-spiroketal spirofungins, macrolide cineromycins, and oligomycin A, antimycin type kitamycins, aflatoxin inhibitor aflastatins, aminoacetophenone family heptaene antibiotics, and novel nikkomycin analogs were found to have potent antifungal activity (Bormann et al. 1999; Hayashi and Nozaki 1999; Holtzel et al. 1998; Kim et al. 1999b; Ono et al. 1998; Schiewe and Zeeck 1999; Vertesy et al. 1998).

Streptomyces have the ability to synthesize diverse compounds covering the chemical structures generated by

the eukaryotic organisms such as fungi, algae, and plants. *Streptomyces kurssanovii* was found to have the ability to synthesize fumaramidmycin, which is structurally very similar to fumarimid and coniothriomycin produced by the fungi *Sordaria* sp. and *Conithyrium* sp. (Maruyama et al. 1975). Although the frequency of rediscovery of known compounds is relatively high, it should also be noted that *Streptomyces* strains continue to provide a larger number and wider variety of new antibiotics than any other microbial sources (Okami and Hotta 1988). Many macrolide antibiotics, for example, have already been introduced from a variety of *Streptomyces* spp., however, new macrolide compounds are still being discovered to be potent antifungal agents. Faeriefungin, a polyene type macrolide, isolated from *S. griseus* showed strong *in vivo* activity against asparagus (*Asparagus officinalis* L.) pathogens *Fusarium oxysporum* and *Fusarium moniliforme* (Smith et al. 1990). More recently, the antifungal substances, phenylacetic acid and sodium phenylacetate, active against *Phytophthora capsici* and *M. grisea* were isolated from the culture filtrates of *S. humidus* (Hwang et al. 2001).

Streptomyces is a sole microbial source for a certain type of antibiotics such as members of manumycin type that contain a multifunctional mC₇N unit as a central structural element. A manumycin type antibiotic SW-B has recently been purified from the culture of *Streptomyces flaveus* strain A11 (Hwang et al. 1996). The strain was isolated from cave soil in Korea by an extensive screening program for the *Streptomyces* strain antagonistic to *P. capsici*. The structure of manumycin SW-B was determined to be 2,4,6-trimethyl deca-(2*E*,4*E*)-dienamide (molecular formula C₁₃H₂₃NO) with the molecular weight of 209.178 (Figure 2). SW-B showed a high level of inhibitory activity and broad antifungal spectrum against several plant pathogenic oomycete and fungi such as *P. capsici*, *M. grisea*, *Colletotrichum cucumerinum*, and *Alternaria mali*. Hyphal growth of *P. capsici* and *M. grisea* was inhibited by more than 50% at 10 µg ml⁻¹ and by 90% at 50 µg ml⁻¹. The simplicity of the chemical structure and its broad antifungal spectrum provide the possibility as a lead compound for fungicide development.

3.2.2 Rare Actinomycetes, New Resource of Microbial Metabolites

Since rare actinomycetes have the properties such as slow growth, poor sporulation, and instability in preservation, it seems difficult to isolate them without applying the selective isolation methods. Most of their metabolites, therefore, were not subjected to the antifungal screening. However, although the antifungal agents from these non-*Streptomyces* groups of actinomycetes have not yet been developed into commercial fungicides, they are expected to be useful microbial sources for diversifying chemical library of metabolites.

The genus *Micromonospora*, only a minor component in the actinomycete population in soil, has been recognized as one of the important sources for antimicrobial metabolites.

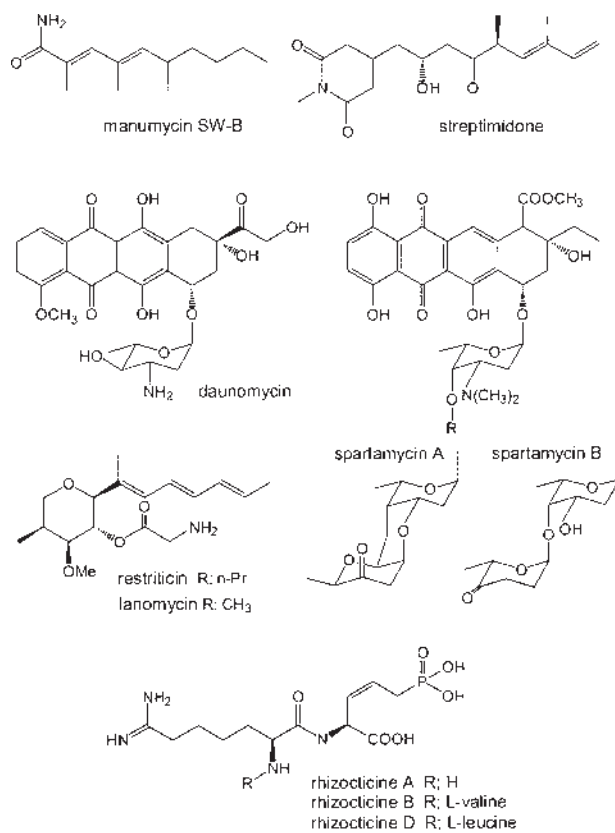


Figure 2 Potential antifungal leads from microbial sources.

Micromonospora spp. was known to be distributed widely in soils of various geographical regions (Vobis 1991). Since gentamicin, an aminoglycoside antibacterial antibiotic, was isolated from *M. purpurea* and *M. echinospora* (Weinstein et al. 1964), *Micromonospora* spp. has been shown to produce diverse antibiotic substances such as aminoglycosides and macrolides (Betina 1994). In a screening program for antifungal antibiotics useful for plant disease control, *Micromonospora coerulea* strain Ao58 was isolated from sea-mud soils, which showed strong antifungal activity against *P. capsici*, *M. grisea*, *C. gloeosporioides*, and *R. solani* (Kim et al. 1998; Kim et al. 1999a). From the culture extracts, the antibiotic streptimidone (Figure 2) was purified using various chromatographic procedures. Streptimidone was known as an inhibitor of the protein synthesis on yeast, but little has been known about its efficacy as an antifungal agent against filamentous fungi. In the tests for antifungal spectrum, remarkable antifungal activities were observed against some plant pathogenic fungi *P. capsici*, *M. grisea*, *Didymella bryoniae*, and *B. cinerea*. *In vivo* tests showed its potent control efficacy against phytophthora blight on pepper plants, gray mold on cucumber leaves, and leaf blast on rice leaves. The compound effectively inhibited the development of these plant diseases on their host plants at the concentration of

100 $\mu\text{g ml}^{-1}$, at which the commercial fungicides showed similar control efficacy against the diseases. No phytotoxicity was observed on any of the host plants at the concentrations of 500 $\mu\text{g ml}^{-1}$.

Recently, two structurally related compounds isolated from rare actinomycetes were found to have potent antifungal activity against plant pathogenic fungi. Daunomycin and spartamycins were isolated from *Actinomadura roseola* and *Micromonospora spartanea*, respectively (Kim et al. 2000b; Nair et al. 1992). Both compounds have similar anthracycline aglycone moiety attached to one or three glycosides (Figure 2). Daunomycin noted for anticancer activity showed substantial *in vitro* antimicrobial activity against *P. capsici*, *R. solani*, *B. cinerea*, *Cladosporium cucumerinum*, *Cylindrocarpon destructans*, *D. bryoniae*, *S. cerevisiae*, and Gram positive bacteria. In particular, daunomycin showed strong inhibitory effect on the mycelial growth of *P. capsici* and Phytophthora development on pepper plants. *In vivo* efficiency against Phytophthora infection in pepper plants was somewhat less effective than that of the commercial fungicide metalaxyl.

Spartamycins produced by *M. spartanea* were isolated from a potted soil with *A. officinalis* L. plants (Nair et al. 1992). Between the two spartamycin analogs A and B, the latter showed better antimicrobial activity against several microorganisms. The minimum inhibitory concentration (MIC) of spartamycin B on *Aspergillus*, *Cladosporium*, *Cryptococcus*, *Rhodotorula*, and *Candida albicans* ranged from 0.2 to 1 $\mu\text{g ml}^{-1}$. However, spartamycin B was not effective against the *Staphylococcus aureus*, *Escherichia coli* and *Citrobacter* spp. In view of antifungal activity and structural similarity of the anthracycline antibiotics, their analogs having different glycoside moieties may be worthwhile to examine their antifungal activity against various plant pathogenic fungi.

3.2.3 Other Microorganisms

Pseudomonas aeruginosa strain B5 was isolated from pepper-growing soils in Korea, which showed substantial inhibitory activity against *P. capsici* and other plant pathogenic fungi. From the culture broth of the antagonistic bacterial strain B5, one of the antibiotic substances active against *P. capsici* was purified and identified as a glycolipid antibiotic rhamnolipid B (Kim and Hwang 1993). Rhamnolipids containing rhamnose and β -hydroxy-decanoic acid were first found in *Pseudomonas pyocyanea* (the old name of *P. aeruginosa*) (Bergström et al. 1946). Recently, complete nuclear magnetic resonance signal assignments of rhamnolipid B based on intensive spectral analysis provided the evidence of 1,2-linkage of 3-[3-[L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-rhamnopyranosyloxy]-decanoyloxy]-decanoic acid (Moon et al. 1996). The glycolipid antibiotic rhamnolipid B has the characteristic structure of biosurfactants, which is comprised of a hydrophilic portion (rhamnose moiety) and a hydrophobic portion (β -hydroxydecanoate moiety). The biosurfactant property was supposed to render the

rhamnolipid the ability to intercalate into and to disrupt the zoospore plasma membrane, because zoospores are surrounded only by plasma membrane without typical cell wall (Stanghellini and Miller 1997). This hypothesis is well supported by the further finding that rhamnolipid B had no lytic activity on zoospore cysts surrounded with the cell wall (Kim et al. 2000a). *In vitro* growth inhibition assay performed in the microtiter dishes showed potent antifungal activities against *Cercospora kikuchi*, *C. destructans*, *C. cucumerinum*, *Colletotrichum orbiculare*, *M. grisea*, and *P. capsici*. In particular, rhamnolipid B had a high level of antifungal activity (10 $\mu\text{g ml}^{-1}$ of MIC) against *P. capsici*. In the microscopic study, most of the zoospores became non-motile in the presence of 25 $\mu\text{g ml}^{-1}$ of rhamnolipid B, subsequently lysing within 1 min after treatment. Rhamnolipid B also was effective in inhibiting the germination of zoospore and the hyphal growth of *P. capsici*. The average hyphal length of germlings at the 50 $\mu\text{g ml}^{-1}$ was reduced by 55% of that in the untreated control. These results suggest that rhamnolipid B has not only the lytic effect on zoospores of *P. capsici* but also inhibitory effect on the growth of the oomycete. Zoospores have been implicated in the spread of the oomycete pathogen through irrigation water and rainwater (Hwang and Kim 1995b; Ristaino et al. 1993). The lytic effect of rhamnolipid B on zoospores may provide a merit as a preventive control agent against phytophthora blight in pepper-growing fields, which eliminate and/or reduce zoospore density and long-distance dispersal of the pathogen. In the recirculating hydroponic cultural system of crops, rhamnolipid B has been demonstrated to be very effective in controlling the dispersal of plant diseases caused by zoosporic oomycete pathogens (Stanghellini et al. 1996).

Bacillus subtilis is known to produce diverse antifungal peptides represented by inturins. A series of fungicidal metabolites, named rhizocticines, were identified from *B. subtilis* ATCC6633 (Figure 2) (Fredenhagen et al. 1995). These peptides showed control efficacy against *B. cinerea* on apples and vines in the greenhouse. The proteolytic digestion test of the compound revealed that L-2-amino-5-phosphono-3-(Z)-pentenoic acid was the actual structure active against *B. cinerea*. The antifungal activity was proven to be stereo specific, since the corresponding 3-(E) compound did not show any antifungal activity. The mixture of rhizocticines A, B, and D also showed control efficacy against gray molds on grapes in the field.

4 POTENTIAL TARGETS FOR DISCOVERY OF ANTIFUNGAL LEADS

Unlike the arena of development of antibacterial agents, in relative terms, where bacterial specific targets are abundant, it seems difficult to develop antifungal agents with a specific mode of action. Since, fungi as eukaryotic organism, have metabolism similar to those of mammal and plant hosts, most

of antifungal agents discovered to be potentially active against plant pathogenic fungi have failed to survive during the testing process for practical usage. The following discussions will be of potential antifungal leads directed to fungal specific targets, with the examples of antifungal agents recently developed for clinical and agricultural uses.

4.1 Cell-Wall Biosynthesis

Fungal cell wall is a crucial target for antifungal agents. Enzymes responsible for the biosynthesis of fungal cell wall include chitin and glucan synthases (Douglas et al. 1997; Georgopadakou 1997). Antifungal agent echinocandins have been discovered as inhibitors of fungal cell wall biosynthesis (Denning 1997). They are noncompetitive inhibitors of β -1,3-glucan synthase, an enzyme complex in the cell wall of many pathogenic fungi. β -1,3-Glucan synthase is a fungal specific enzyme that polymerizes UDP-glucose into β -1,3-glucan polymers that comprise the major scaffolding of the fungal cell wall (Kang and Cabib 1986). Echinocandins and its synthetic analog containing the fatty acid side chain, cilofungin, exhibited comparable fungicidal activity with a narrow antifungal spectrum (Fromtling 1994). Recently, marked improvements in antifungal activity against clinical pathogens have been achieved by synthetic variations made in the lipid side chains of echinocandins. LY30336 and caspofungin are the examples of recently developed echinocandin analogs (Espinel-Ingroff 1998), which are generally more active *in vitro* against a variety of yeast and filamentous fungi. They are licensed by Lilly and Merck, respectively, for clinical usage. Such a novel mechanism of action, antifungal potency and relatively broad-spectrum activity of echinocandins provide the possibility that the inhibitors of β -1,3-glucan synthase may be available for the development of biofungicides effective against fungal diseases (Pfeller et al. 1998).

Chitin, β -1,4-*N*-acetylglucosamine polymer, plays a major structural and strengthening role in fungal cell walls. Chitin is microfibrills consisting of hydrogen-bonded polysaccharide chains that may be covalently cross-linked to other polysaccharide, mainly glucan. It has been demonstrated that chitin synthase inhibitors and chitinase showed antifungal activity when applied to growing cells (Lorito et al. 1993). Nikkomycins are analogs of UDP-*N*-acetylglucosamine produced by *Streptomyces* spp. They have potent activity against chitin synthase by acting as specific competitive inhibitors (Hunter 1995). The potency of an inhibitor of chitinase synthase may depend on not only the isoform's relative effectiveness in building a cell wall, but also its affinity to a given enzyme. Recent research on chitin synthase revealed that the multiple chitin synthase genes of fungi have different sensitivities to the inhibitors (Munro and Gow 1995). Therefore, new antifungal compounds with higher activity and specificity to chitin synthase may be generated from diverse chemical pool of microbial metabolites.

4.2 Sterol Biosynthesis

The biosynthesis of sterols is an essential metabolism that produce essential constituents of cellular membranes. Most of fungi contain ergosterol as a predominant sterol (Mercer 1991). Recent advances in our understanding of mode of action of sterol biosynthesis inhibitors (SBI) launched a novel approach to finding inhibitors of sterol biosynthesis, which could lead to new agricultural fungicides (Barrett-Bee and Ryder 1992). The antifungal effects of SBI have brought out a great commercial success in the synthetic fungicide market. The SBI fungicides covering about the half of the market is now practically applied to protect fruits, vegetables, and vines from plant diseases. The major SBI are the inhibitors of 14-demethylation which correspond to many antifungal compounds, referred to as azole compounds, with a wide spectrum of intrinsic activity against ascomycete, basidiomycete and deuteromycete pathogens (Aoki et al. 1993).

The discovery of restricticins and lanomycin led to the introduction of a new target for screening of the antifungal natural products. The two structurally related compounds were first isolated from the cultures of *Penicillium restrictum* (Schwartz et al. 1991) and *Pycnidophora dispersa* (O'Sullivan et al. 1992), respectively (Figure 2). Both restricticin and lanomycin showed potent antifungal activity through inhibition of lanosterol C₁₄-demethylase, one of the main steps in ergosterol biosynthesis. It is interesting to note that the structure of restricticin does not have a (phenylethyl)triazole moiety found in all azole antifungal agents in the market, possibly causing adverse impacts in efficacy and resistance (Tuite 1996). However, restricticin needed to be improved in its chemical stability, because the compound was found to be unstable due to the lability of the glycine ester side chain toward base-mediated hydrolysis and the tendency of the triene functionality to undergo decomposition (Barrett-Bee and Ryder 1992). Along with the advances in the screening for inhibitors of other steps in sterol biosynthesis, more SBI sufficient for practical uses may be discovered from microbial metabolites.

4.3 Acetyl-CoA Carboxylase (ACC)

Discovery of soraphen A from myxobacteria was an important event in antifungal metabolite development, because of not only enlarging microbial diversity as a source of antifungal compounds but also introducing fungal ACC as a novel target for antifungal agent screening (Gerth et al. 1994). Acetyl-CoA carboxylase catalyzes carboxylation of acetyl-CoA to malonyl-CoA at the expense of ATP. While the functional units of ACC are usually separate proteins in prokaryotes, they form a multifunctional enzyme complex in eukaryotes. This may be the reason why soraphen A is inactive to bacteria. Soraphen A which is mainly responsible for antifungal activity of *Sorangium cellulosum* strain Soce26 effectively controlled powdery mildew (*Erysiphe graminis*

f. sp. *hordei*) in barley, snow mold (*Gerlachia nivalis*) in rye, apple scab (*Venturia inaequalis*) on apple and gray mold (*B. cinerea*) on grape (Reichenbach and Höfle 1995). Soraphen A has no effect on ACC of plants, thus inducing no phytotoxicity in the field (Vahlensieck et al. 1994). In contrast, ACC from rat liver was strongly inhibited by the soraphen (Pridzun et al. 1995). Due to the risky side effects on experimental animals, soraphen A has not been practically used for control of plant diseases. However, the results of soraphen research strongly suggest that fungal ACC could be a target site for antifungal agent screening. Considering the numerous diversity of natural products related to the specificity of ACC, novel biofungicides from microbial metabolites that block specifically the activity of fungal ACC may be developed in the future.

4.4 Nucleic Acid Metabolism

One of the areas that can be exploited as antifungal targets is nucleic acid metabolism. The synthesis of nucleic acids involves numerous biochemical reactions ranging from the initial synthesis of purine and pyrimidine precursors to the final polymerization of ribonucleoside and deoxyribonucleoside 5'-triphosphates into RNA and DNA. A large number of compounds have been known to be inhibitors of nucleic acid metabolism in fungi. However, few of these compounds have been used as agricultural and clinical antifungal agents. Recently, antibiotic tubercidin produced by *Streptomyces violaceoniger* was discovered to have antifungal activity against plant pathogenic fungi (Hwang and Kim 1995a; Hwang et al. 1994). It was highly active against *P. capsici*, *Botryosphaeria dothidea*, and *R. solani*. Tubercidin is an adenosine analog that interferes nucleic acid synthesis including *de novo* purine synthesis, rRNA processing, and tRNA methylation (Suhadolnik 1979). The potent *in vivo* activity of tubercidin against *P. capsici* was compared with that of systemic fungicide, metalaxyl, which is one of the best-studied acylalanine targeting on the synthesis of ribosomal RNA. Treatment with tubercidin on day 1 before inoculation of zoospores prevented phytophthora blight at 500 $\mu\text{g ml}^{-1}$. Tubercidin was effective as much as metalaxyl, irrespective of application time and concentrations, although its antifungal activity did not persist as long as metalaxyl in pepper plants. The potent antifungal activity of tubercidin against *P. capsici* suggests that possible targets for the antifungal agent screening may be present in nucleic acid metabolic pathway.

4.5 Protein Biosynthesis

Protein biosynthesis is available as a set of molecular targets for antibacterial agent development. The antibiotics such as chloramphenicol and streptomycin have been demonstrated to inactivate or alter the accuracy of the bacterial ribosome (Cundliffe 1990). However, the use of fungal protein

biosynthesis as an antifungal target has been more challenging, because of the high degree of structural and functional identity of the components of the protein biosynthetic machinery between fungi and higher eukaryotes. As in the cases of cycloheximide, trichodermin, and hygromycin B, their activities on the fungal ribosome appear to be identical to those on the mammalian ribosome (Tuite et al. 1995). It was, therefore, of considerable interest to find out a specific antifungal agent targeting on fungal protein biosynthesis.

Sordarins were found to have highly specific inhibitory activity against the elongation factor 2 involved in the translation of several fungal species (Justice et al. 1998). Sordarins were originally isolated from the terrestrial ascomycete *Sordaria araneosa* (Hauser and Sigg 1971). The fungal specific activity of sordarins is quite interesting, because the elongation factor 2 is a highly conserved protein. Recently, a mutant strain analysis revealed that sordarins had additional interactions with the ribosome itself (Justice et al. 1999), indicating that the selectivity of these compounds was governed by multiple points of interaction between the compound and the ribosome. Using a high throughput screening (HTS) targeting on protein synthesis in *Candida* spp., an analog of sordarin has recently been demonstrated to be an effective *in vitro* inhibitor with apparent selectivity for fungal protein synthesis (Kinsman et al. 1998).

5 FUTURE TRENDS IN BIOFUNGICIDE RESEARCH

During the last two decades, there were numerous efforts focusing on the isolation and identification of a wide range of biologically active natural products. As a result, hundreds of thousands to millions of compounds became available for the evaluation of their value as potential lead compounds. The concept of a HTS was developed to screen a large number of chemical libraries, which overcome the limitation of conventional *in vitro* and *in vivo* assay. The HTS is made possible by the advance in assay system, which was designed to target a specific biochemical event in fungal metabolism. A direct measure of the activity of the compound at the target of interest can be done without complications arising from other metabolic events. These approaches can enhance the possibility to discover new and useful biofungicides by supplying unique bioassay system. This innovative procedure was already applied in developing new fungicides such as sordarins mentioned earlier. The target-directed screening will be fortified by DNA sequence information that is exponentially increased in recent years by a number of fungal genome projects. The genomic information can provide a wealth of new targets to be validated and screened for new antifungal leads (DiDomenico 1999).

Along with the innovations in screening systems, the efforts to diversify the chemical library of microbial metabolites has been continued through combinatorial

approaches. Recently, the ability to synthesize a large number of chemical libraries from core structure of antibiotics was greatly enhanced by the advance of rapid combinatorial/parallel synthesis method (Caporale 1995). The diversity and numbers of distinct compounds in combinatorial library enhance the possibility of finding a chemical structure with the desired properties. Combinatorial libraries can be synthesized in many different ways as reviewed by Dolle (1999). However, most of the successes in combinatorial chemistry have been accomplished by using small libraries to improve the properties of a specific toxophore. The successful optimization of azole and oxazolidinone lead compounds suggested a promising future of combinatorial chemistry in biofungicide research (Trias 2001).

As another approach to diversify the chemical library of microbial metabolites, combinatorial biosynthesis was proposed to generate "unnatural" natural products, which use genetic information and DNA recombination techniques to alter the biosynthetic pathway of the microorganism to produce the designed chemical structure. This can also be done by introducing hybrid enzyme or/and swapping with heterologous biosynthetic machinery involved in the synthesis of other antibiotics (Cropp et al. 2002; Reynolds 1998). More recently, previously unknown chemical structures were generated by interchanging enzyme subunits or making hybrid enzymes of type I polyketide synthases (PKSs) (Kim et al. 2002; McDaniel et al. 1999; Yoon et al. 2002). Most of the combinatorial biosynthesis researches have been done on PKSs, especially in *Streptomyces*. Recently, the biosynthetic gene clusters of antifungal antibiotic pyoluteorin and 2,4-diacetylphloroglucinol also were identified from plant-associated pseudomonads, the well-known biological control agents (Bender et al. 1999). These biosynthetic gene clusters are expected to be used for the template of combinatorial biosynthesis for biofungicide development, although a number of questions about their enzymological functions still remains to be elucidated.

6 CONCLUSIONS

As the environmental and commercial requirements for new fungicides become more demanding, the merits of biofungicides over synthetic fungicides become more important than ever. Recently, a breakthrough in biofungicide research was made by semisynthetic approaches using antifungal microbial metabolite as the starting point. As seen in the examples of fenpiclonil, fludioxonil and synthetic derivatives of antibiotic strobilurins such as β -methoxyacrylate azoxystrobin and kresoxim-methyl, this approach is a promising and effective strategy for the development of new biofungicides with desired chemical and biological characteristics. These successes encourage fungicide researchers to construct versatile chemical library of microbial metabolites that can be used for development of new fungicides. Recently, a number of antifungal compounds have been discovered from diverse microbial sources including *Streptomyces*, rare

actinomycetes, other eubacteria and fungi, which may be available for antifungal leads. The advances in the screening system directed to fungal specific targets have rendered more chances to get success in biofungicide development. A number of useful targets have been discovered from the fungal metabolism related to nucleic acid, protein, sterol, and cell-wall biosynthesis. The recent successful example of sordarin analogs show that better understanding of biochemical events in fungal cells would uncover more useful targets for the screening of antifungal leads. Combinatorial approaches in chemical and biochemical synthesis were suggested to diversify the chemical library of microbial metabolites, which can make it easier to discover the optimized antifungal compound with desired physical and biological properties. These new trends in developing novel biofungicides will be more facilitated and strengthened by innovative multidisciplinary approaches in the future.

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Molecular Biology of Biocontrol *Trichoderma*

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1 INTRODUCTION

Plant diseases, caused primarily by fungal and bacterial pathogens, produce severe losses to agricultural and horticultural crops every year. These losses can result in reduced food supplies, poorer quality agricultural products, economic hardship for growers and processors, and, ultimately, higher prices. For many diseases, traditional chemical control methods are not always economical nor are they effective, and fumigation as well as other chemical control methods may have unwanted health, safety, and environmental risks. Biological control involves the use of beneficial microorganisms to attack and control plant pathogens and the diseases they cause. It offers an environmentally friendly approach to the management of plant disease and can be integrated into an effective integrated disease management system. Thus, biological control can be an important component in the development of a more sustainable agriculture.

Trichoderma species have been investigated as biological control agents for over 70 years, but it is only relatively recently that strains have become commercially available. The previous considerations have stimulated researchers to gain a better knowledge of biocontrol by this fungus, and to understand their mechanisms of control. In view of the actuality of this research field, there are numerous recent articles available which review the current state of knowledge of *Trichoderma* biocontrol (Chet et al. 1998; Harman and Björkman 1998; Hjeljord and Tronsmo 1998; Monte 2001 see also the article by A. Herrera-Estrella and I. Chet, this volume). In this article, the current state of biological knowledge on *Trichoderma* strains capable of biocontrol on a molecular level will be summarized.

2 TRICHODERMA BIOCONTROL TAXA AND STRAINS

The genus *Trichoderma* currently consists of more than 40 known taxa, which are usually cosmopolitan, (although some species display a geographic bias: Kubicek et al. 2002; Kullnig et al. 2000), and typically soilborne or wood decaying. Teleomorphs of *Trichoderma* occur in the genera *Hypocrea*, *Podostroma*, and *Sarawakus* of the *Hypocreaceae* (Gams and Bissett 1998; Rossman et al. 1999). The latter two genera thereby most likely being synonyms of *Hypocrea* (GJ Samuels, personal communication). Rossman (1999) proposed that necrotrophy (on basidiomycetes) is the original habitat of these *Hypocrea* spp. and their lignicolous properties have developed later when the species were following their hosts into their habitat (wood and decaying wood in soil). Rossman et al. (1999) claim that the *Hypocrea* spp., which are found on decaying wood, actually are necrotrophic on the fungi in the wood. Several of the individual teleomorphic and anamorphic partners have been detected recently, and examples relevant to biocontrol are given in Table 1. The more than 100 species of *Hypocrea* with *Trichoderma* anamorphs which Doi and Doi (1986) described constitute unexplored source of potential biocontrol agents.

Most of the isolates of the genus *Trichoderma*, which have been found to act as biocontrol agents, have been classified as *T. harzianum* Rifai, leading to the fact that *T. harzianum* is generally synonymized as a “biocontrol agent.” However, most of the *Trichoderma* strains used for biocontrol were identified at the species level exclusively on the basis of morphological and phenotypical characters, showing high convergence in many cases (Kullnig-Gradinger et al. 2003). Therefore, reports of a pronounced genetic variability of *T. harzianum* isolates by analyzing carbon

Table 1 Teleomorphs known for *Trichoderma* taxa used in biocontrol

Anamorph	Teleomorph
<i>T. harzianum</i>	<i>H. lixii</i> (former <i>H. nigricans</i>)
<i>T. atroviride</i>	<i>H. atroviride</i>
<i>T. virens</i>	<i>H. virens</i>
<i>T. asperellum</i>	Not known
<i>T. parceramosum</i>	Not known
<i>T. longibrachiatum</i>	Not known

source utilization patterns (Manczinger and Polner 1985), secondary metabolite production (Okuda et al. 1982), isoenzyme polymorphism (Grondona et al. 1997; Stasz et al. 1989), RAPD profiles (Fujimori and Okuda 1994; Gomez et al. 1997; Muthumeenakshi et al. (1994); Turoczi et al. 1996; Zimand et al. 1994), RFLP patterns (Bowen et al. 1996; Muthumeenakshi et al. 1994), rDNA sequence (Grondona et al. 1997; Muthumeenakshi et al. 1994) and karyotype (Gomez et al. 1997) must be treated with caution. On the basis of a rigorous comparison of a pool of seventeen bonafide "*T. harzianum*" biocontrol strains with the neo-*ex* type strain of *T. harzianum*, Hermosa et al. (2000) showed that they actually comprised of four different species i.e., *T. harzianum*, *T. atroviride*, *T. longibrachiatum* and *T. asperellum*. Consistent results were also reported by Kullnig (2001), who by sequence analysis of the internally transcribed spacer regions of the rDNA (ITS1 and ITS2), the small subunit of the mitochondrial DNA (mtSSUrDNA), and part of the coding region of the 42-kDa endochitinase encoding gene *ech42*- reassessed the species identity of eight *T. harzianum* isolates, which are being used by several laboratories for key investigations on the genetics, biochemistry, and physiology of biocontrol. Thereby the strains *T. harzianum* CECT 2413, T-95, T-22, and T-11 were confirmed as *T. harzianum*, "*T. harzianum*" ATCC 74058, IMI 206040, ATCC 36042 identified as *T. atroviride*, and "*T. harzianum*" T-203 assessed as *T. asperellum*. As outlined above, there may be other species capable of biocontrol as well, *T. virens* being the most prominent example. In addition, molecular proof for identity of other species as biocontrol agents has been presented for *T. ghanense* (previously *T. parceramosum*; Arisan-Atac et al. 2002) and *T. stromaticum* (Samuels et al. 2000).

3 IN SITU MOLECULAR TOOLS FOR BIOCONTROL STRAINS

Even if the species identity is not a concern, the ability to recognize the strain which was introduced into the field is of interest. Appropriate molecular tools have thus recently been introduced for identifying *Trichoderma* strains in the environment, and to follow their fate after introduction into

the soil *in situ*. To monitor the behavior of a given strain in the soil, Bae and Knudsen (2001) cotransformed *T. harzianum* with genes encoding green fluorescent protein (*GFP*), beta-glucuronidase (*GUS*), and hygromycin B resistance (*hygB*). One of the resulting strains was formed into calcium alginate pellets and placed onto buried glass slides in a nonsterile soil, and its ability to grow, sporulate, and colonize sclerotia of *Sclerotinia sclerotiorum* was compared with that of the wild-type strain. The green color of cotransformant hyphae was clearly visible with a UV epifluorescence microscope, while indigenous fungi in the same samples were barely visible. Green-fluorescing conidiophores and conidia were observed within the first 3 days of incubation in soil, and this was followed by the formation of terminal and intercalary chlamydospores and subsequent disintegration of older hyphal segments. In addition, no significant differences were detected in colonization levels between wild-type and cotransformant strains; and the authors concluded that GFP proved a most useful tool for nondestructive monitoring of the hyphal growth of the transformant in a natural soil. Also, the chromogenic substrate 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) could be used to monitor the activity of β -glucuronidase in soil. Thus, cotransformation with GFP and GUS can provide a valuable tool for the detection and monitoring of specific strains of *T. harzianum* released into the soil.

As the biological strains of *Trichoderma* are difficult to distinguish from the indigenous strains of *Trichoderma* found in the field, Hermosa et al. (2001) developed a method to monitor these strains when applied to natural pathosystems. To this end they used random amplified polymorphic DNA (RAPD) markers to estimate genetic variation among sixteen strains of the species *T. asperellum*, *T. atroviride*, *T. harzianum*, *T. inhamatum*, and *T. longibrachiatum*. Analysis of the respective RAPD products generated were used to design specific primers. Diagnostic PCR performed using these primers specifically identified one of their strains (*T. atroviride* 11), and clearly distinguished this strain from other closely related *Trichoderma* isolates, showing that SCAR (sequence-characterised amplified region) markers can be successfully used for identification purposes.

An alternative approach, suitable to monitor the presence of several strains in one sample was presented by van Elsas et al. (2000) by selecting a nested PCR approach, in which the first PCR provided the required specificity for fungi, whereas the second (nested) PCR served to produce amplicons separable on denaturing gradient gels. Denaturing gradient gel electrophoresis (DGGE) allowed the resolution of mixtures of PCR products of several different fungi including *Trichoderma*. Although only limited examples have so far been published, techniques like these and the fast current advance in PCR technology (such as real-time PCR to name only one) will stimulate further studies of the behavior of *Trichoderma* biocontrol agents in the field is now possible.

4 GENOME ORGANIZATION AND REPRODUCTION

One of the major difficulties with *Trichoderma* biocontrol strains is their genetic instability, whose reason is only poorly understood at present. This is in part due to the fact that only little is known about the genome organization and its plasticity of *Trichoderma*. Not even the number of chromosomes is known with certainty: Fekete et al. (1996) separated six chromosomes in five *Trichoderma* biocontrol strains with sizes ranging from 3.7 to 7.7 Mb; estimated genome sizes were between 30.5 and 35.8 Mb. When fractionated chromosomes of the five species were probed with a fragment of the *ech42* (endochitinase-encoding) gene, strong hybridization signals developed, but their physical position varied among species indicating a polymorphic chromosomal location. Herrera-Estrella et al. (1993) compared the molecular karyotype of *T. reesei* with that of *T. atroviride* (named erroneously *T. harzianum* in their study), and *T. viride*, and detected largely similar chromosomal organization of genes in different species, although *T. viride* seemed to lack the smallest chromosome. Similarly, Hayes et al. (1993), when karyotyping three biocontrol strains of *T. harzianum* (one parent and two mutants derived from it), found that the smallest chromosome was not present in the mutants. While all these studies revealed a low degree of chromosome polymorphism at the species level, the karyotypes were relatively constant. A report to the contrary (Gomez et al. 1997) is probably flawed by the use “*T. harzianum*” strains which in fact consisted of several different species (CP Kubicek, unpublished data). Thus, as expected for an asexual fungus, chromosome plasticity is unlikely responsible for the genetic instability of *Trichoderma* biocontrol strains.

Molecular genetic work with *Trichoderma* spp. is still limited by the only rudimentary information about its genomic organization as is available for *Aspergillus fumigatus* (<http://www.tigr.org/tdb/e2k1/afu1/>) and *Neurospora crassa* (<http://www-genome.wi.mit.edu/annotation/fungi/neurospora>). Genetic maps could so far not be constructed, because the teleomorphs of biocontrol species of *Trichoderma* (see Table 1) do not cross in axenic culture (CP Kubicek, unpublished data). Also, at the time of this writing, genome sequencing projects on selected species of *Trichoderma* have only just been initiated at a few places, and no results from these are yet available. However, a collection of 1151 ESTs of *T. reesei* grown on glucose and the sequence of the complete mitochondrial genome is already available in the Internet (<http://trichoderma.iq.usp.br/TrEST.html>), and can (because of the high similarity of nucleotide sequences of protein encoding genes within the genus (unpublished data)) be used for picking genes from biocontrol strains as well).

Interestingly, Seiboth and Hofmann (2002) found a similar genomic organization of several genes of galactose metabolism in *T. reesei* and *N. crassa*. This finding is highly

interesting, as *N. crassa* has evolved about 200 million years ago (Berbee and Taylor 1993), whereas *H. jecorina* evolved only about 100 million years ago (Kullnig-Gradinger et al. 2003), and thus the genomic organization of these genes has been maintained constant for about 100 million years. Hamer et al. (2001) have also recently reported that a 53-kb region of the genome of *Magnaporthe grisea* was also syntenic to a corresponding portion of the *Neurospora* genome. In a comprehensive study on hemiascomycetous yeasts, Llorente et al. (2000) demonstrated that even phylogenetic distant species such as *S. cerevisiae* and *Yarrowia lipolytica* exhibit 10.1 % of conserved synteny. If there is indeed a high degree of synteny between *Neurospora* and *Trichoderma*, this may be useful for studying the genomic organization of *Trichoderma* biocontrol strains.

Probably due to reproduction, largely via asexual mechanisms, many species of *Trichoderma* reveal a high level of genetic stability (cf. Kubicek et al. 2002; Kullnig et al. 2000). *T. harzianum*, however, is a noteworthy exception, showing a remarkable intraspecific genetic and phenotypic variation, and this may also be related to the instability of the respective biocontrol species. The reason for this has not been explained yet. As the respective teleomorph (*H. lixii*) is known, the possibility of sexual recombination still needs rigorous testing. Transposons, which have been isolated from phylogenetically close fungal genera such as *Tolypocladium* or *Fusarium*, are another possibility. We have recently observed a very high noninduced mutation rate in one biocontrol strain of *T. harzianum* which would be compatible with the presence of a mobile element (C Gallhaup, RL Mach and CP Kubicek, unpublished).

As far as nonchromosomal elements are concerned, plasmids have been detected in filamentous fungi almost exclusively in the mitochondrion (Bertrand 2002). They are generally stable genetic elements and vary between 1–6 kb size. In accordance with this situation, Meyer (1991) detected mitochondrial plasmids in strains of *T. viride* and the biocontrol-relevant species *T. asperellum* (then named “*T. viride* 2”). A circular plasmid called pThr1, with a monomer size of 2.6 kb, was identified in the mitochondria of the biocontrol isolate *T. harzianum* T95 (Antal et al. 2002). It revealed no DNA sequence similarity with the mitochondrial genome of the isolate and contained a single 1818 bp open reading frame. The derived amino acid sequence exhibited similarity to the reverse transcriptases of the circular Mauriceville and Varkud retroplasmids of *Neurospora* spp. and the linear pFOXC2 and pFOXC3 retroplasmids of *Fusarium oxysporum* strains. In the regions of homology all of the seven conserved amino acid blocks characteristic of RTs could be found. In *Fusarium oxysporum* f. sp. *conglutinans*, these mitochondrial plasmids have been identified as factors determining the host specificity (Kistler and Leong 1986); unfortunately, corresponding investigations are still lacking for *Trichoderma*.

5 MOLECULAR GENETIC BASIS OF BIOCONTROL

Arising from necrotrophic ancestors, most of the currently known *Trichoderma* strains have developed highly effective antagonistic mechanisms to survive and colonize the basidiomycete-containing competitive environment of the rhizosphere, soil, and decaying wood. Active parasitism on host fungi, by penetration of host hyphae is probably the mechanism most studied (cf. Chet et al. 1998); it requires morphological changes of *Trichoderma* hyphae such as appressorium formation and coiling, and is further supported by the production of extracellular enzymes, and production of antifungal antibiotics. However this mechanism has mostly been observed in the laboratory, and application of *Trichoderma* in the field may involve additional mechanisms as well such as aggressive degradation of organic matter, thereby competing for nutrients which in saprobic phases may be a limiting factor. Also promotion of the growth and biological activities of saprobic bacteria and mycorrhizal fungi, and of plant-growth and induced resistance have been reported (for review see Herrera-Estrella and Chet, Chapter 57).

Among these, mycoparasitism is the only process which has been studied on a molecular biological basis. Herrera-Estrella and Chet (Chapter 57) give a detailed account on this, and I shall therefore treat this point here only very briefly: most attention has been paid to the enzymatic disruption of the cell wall of the fungus, thereby focusing on enzymes capable of hydrolysis its structural polymers (chitin, β -glucan, protein and others). Genes encoding endochitinases, *N*-acetyl- β -glucosaminidases, proteases, endo- and exo-glucan β -1,3-glucosidases, endoglucan- β -1,6-glucosidases, lipases, xylanases, amylases, phospholipases, RNases, and DNases have been cloned from various biocontrol species of *Trichoderma*, and are listed in detail in the above-mentioned chapter (also see Benitez et al. 1998; Kubicek et al. 2001; Lorito 1998). Most of these enzymes showed very strong antifungal activity against a variety of plant pathogenic fungi *in vitro*. Several of these cell wall degrading enzymes, but most notably chitinases, have thus been demonstrated to have a great potential as active components in new fungicidal formulations or genetically modified plants.

Interestingly, the endochitinases found in *Trichoderma* belong only to one (class V) of the several classes of the chitinases known from plants (Beintema 1994). The latter show a modular structure, and frequently contain protein domains capable of binding to chitin, which bear some resemblance to the cellulose-binding domains also found in *Trichoderma* cellulases. In contrast, none of the chitinases cloned from *Trichoderma* spp. so far has been shown to contain such a chitin-binding domain. To investigate the role of the latter, Limon et al. (2001) have produced hybrid chitinases with stronger chitin-binding capacity by fusing to Chit42 a ChBD from *Nicotiana tabacum* ChiA chitinase and

the cellulose-binding domain from cellobiohydrolase II of *T. reesei*. The chimeric chitinases had similar activities as the native chitinase towards soluble substrates, but higher hydrolytic activity on high molecular mass insoluble substrates (chitin or fungal cell walls). Unfortunately, no results from *in vivo* biocontrol tests were reported, and it remains thus unclear whether the presence of such a domain would improve the antagonistic abilities of *Trichoderma* biocontrol strains.

The action of chitinases and glucanases is also strongly synergistic both with other chitinase components as well as with other components putatively involved in biocontrol, i.e., antibiotics (Jach et al. 1995; Lorito et al. 1994; 1996b; Schirmböck et al. 1994). In the case of the peptaibols, the mechanism of this enzyme-antibiotic synergism has been shown to be due to a synergistic effect of enzyme and the antibiotic on the maintenance of cell wall integrity (Lorito et al. 1996b). Peptaibols are linear oligopeptides of 12–22 amino acids, which are rich in α -aminoisobutyric acid, *N*-acetylated at the N-terminus and containing an amino alcohol (Pheol or Trpol) at the C-terminus (Rebuffat et al. 1989), and known to form voltage-gated ion channels in black lipid membranes and modify the membrane permeability of liposomes in the absence of applied voltage (El Hadjji et al. 1989). Hence, while the chitinases reduce the barrier effect of the cell-wall, peptaibol antibiotics inhibit the membrane bound chitin- and β -glucan synthases and thereby impair the ability of the hyphae to repair the lytic effect of the enzymes on the cell walls polymers.

The gene (*tex1*) encoding the enzyme synthesizing these peptaibols (peptaibol synthase) has recently been cloned from *T. virens* (Wiest et al. 2002). It comprises a 62.8 kb continuous open reading frame encoding a protein structure consisting of 18 peptide synthetase modules with additional modifying domains at the N- and C-termini. Mutation of the gene eliminated production of all peptaibol isoforms, indicating that their formation is due to a relaxed substrate specificity of the individual synthase domains. Interestingly, the nucleotide sequence of *tex1* is 100% identical to a 5,056-bp partial cDNA fragment of another gene (*psy1*) isolated also from *T. virens* (Wilhite et al. 2001). These authors observed that *psy1* disruptants grew poorly under low-iron conditions, and failed to produce the major *T. virens* siderophore, dimerum acid (a dipeptide of acylated *N*(α)-hydroxyornithine, thus suggesting that Psy1 plays a role in siderophore production. Biocontrol activity against damping-off diseases caused by *Pythium ultimum* and *Rhizoctonia solani* was not reduced by the *psy1* disruption. The discrepancy between the results reported by Wiest et al. (2002); Wilhite et al. (2001) need to be explained before the importance of the *tex1/psy1* gene in biocontrol can be estimated.

Peptaibols, however, are certainly not the only secondary metabolites with synergistic action in host cell-wall degradation. Other components (e.g., γ -pentyl pyrone) was also found to be important for antagonism *in vivo* (Claydon et al. 1987; Howell 1998; Serrano-Carreón et al. 1993), and

their mechanism of action thus awaits to be elucidated. 6-pentylpyrone is probably the most frequently studied of these metabolites, as it also exhibits a pronounced “coconut-aroma” which can be used as a (for humans) nontoxic flavoring agent. Its biosynthesis has been claimed to be derived from linolenic acid (Serrano-Carreón et al. 1993), but this conclusion was criticized by Sivasithamparan and Ghisalberti (1998), who consider it to be a product of polyketide biosynthesis. No other of the genes or proteins involved in *Trichoderma* secondary metabolism has as yet been characterized.

6 BIOCONTROL-SPECIFIC GENE EXPRESSION IN *TRICHODERMA*

In the laboratory, high-level induction of extracellular cell-wall lytic enzymes is usually obtained by growing *Trichoderma* on purified chitin, fungal cell walls, or mycelia as sole carbon sources. No, or much less, induction is normally obtained when related compounds such as chitosan, cellulose, unpurified chitin, or laminarin are used. In addition, formation of most chitinolytic enzymes does not occur or is even inhibited by glucose, sucrose, and chitinolytic end-products (Carsolio et al. 1994; García et al. 1994; Lorito et al. 1996a; Margolles-Clark et al. 1996; Peterbauer et al. 1996), suggesting that direct induction and/or catabolic repression are major regulatory parameters for chitinase formation. Some researchers also found trace quantities of some chitinases (e.g. the 102-kDa *N*-acetyl- β -D-glucosaminidase, the 42-kDa endochitinase and the 33 kDa endochitinase) are produced constitutively (Carsolio et al. 1999; García et al. 1994; Haran et al. 1995; Inbar and Chet 1995; Margolles-Clark et al. 1996). It should be noted that this does not rule out regulation of the respective promoters by induction only, due to the fact that the binding of DNA-binding proteins to their target sequences is an equilibrium, every promoter will partially be in transcriptionally active state, depending on the K_d and the concentrations of the respective proteins.

Some of these findings have recently been supported by the analysis of gene expression. The expression of *T. atroviride* *nag1* is triggered by fungal (*B. cinerea*) cell walls and the commercially available chitin monomer *N*-acetyl-glucosamine, and the oligomers di-*N*-acetyl-chitobiose and tri-*N*-acetylchitotriose (Mach et al. 1999). In contrast, *ech42* expression in *T. atroviride* was also observed during growth on fungal cell walls, but could not be triggered by those chitin degradation products (Margolles-Clark et al. 1996; Cortés et al. 1998; Mach et al. 1999), whereas in *T. harzianum* it is induced by *N*-acetyl- β -D-glucosamine (García et al. 1994; Schickler et al. 1998). Digestion of the host cell walls with specific combinations of purified *Trichoderma*-secreted chitinases and glucanases (both endo- and exo-acting) released products that strongly elicited *ech42* and *nag1* gene expression and consequent mycoparasitic activity. Lorito (2002) recently reported the purification of

these low-molecular weight, biocontrol-inducing molecules released from the host cell walls, and showed that they were much more active *in vitro* than purified chitin or glucan monomers.

The failure of *T. atroviride* to induce *ech42* expression by chitin may be due to complex interactions within different regulatory circuits (Donzelli and Harman 2001): both *ech42* and *nag1* required both nitrogen starvation and the presence of chitin for induction, whereas *gluc78* could be induced by nitrogen starvation alone. In the presence of low levels of ammonium (10 mM), both chito-oligomers and chitin triggered CHIT42 and CHIT40 (chitobiosidase) production. CHIT73 secretion occurred in the presence of *N*-acetyl-glucosamine and chito-oligomers, while chitin was less effective. These results indicate that the expression and secretion of cell wall-degrading enzymes by *Trichoderma* is nitrogen repressed, and that effects of carbon and nitrogen nutrition are interactive. The expression of *ech42* from *T. atroviride* after prolonged carbon starvation is likely not due to a relieve from carbon catabolite repression, as it can be observed with glucose as well as with glycerol as a carbon source (Mach et al. 1999). In addition, *ech42* gene transcription was triggered by some conditions of physiological stress (4°C, high osmotic pressure, addition of ethanol; Mach et al. 1999), as well as during light-induced sporulation (Carsolio et al. 1994). Interestingly, *T. harzianum* *chit33* expression, while being inducible by *N*-acetyl- β -D-glucosamine, was also triggered by carbon starvation, nitrogen starvation and physiological stress (de las Mercedes Dana et al. 2001), suggesting that stress-mediated regulation may be a general phenomenon involved in chitinase gene expression of *Trichoderma* spp.

Some studies have so far been performed towards understanding how and in which order the chitinases are induced during mycoparasitic interaction. In their pioneering studies, Inbar and Chet (1992); (1995) demonstrated that formation of chitin-degrading enzymes in *T. harzianum* is elicited by a lectin-based physical interaction with the host, which was suggested to be the earliest event of interaction, and precede induction by possible chito-oligomers (see chapter Herrera-Estrella and Chet). Inbar and Chet (1995) showed that a 102-kDa chitinase is specifically induced by contact with the host lectin, whereas formation of all the other chitinases requires the presence of the living host. They concluded that an *N*-acetyl- β -D-glucosaminidase with apparent denatured M_r of 102 kDa may be responsible for the first attack and induction for the other chitinases. However, Zeilinger et al. (1998), using the *Aequorea victoria* GFP as a nondisruptive reporter system, showed that *ech42*, but not *nag1*, was formed before any detectable contact of *Trichoderma* with its host. Similar studies with *chit33::GFP* in *T. harzianum* (de las Mercedes Dana et al. 2001) showed that this pre-contact gene expression did not occur with the 33-kDa endochitinase-encoding gene *chit33*, and therefore may be specific for *ech42*. Interestingly, *ech42* gene expression was prevented when a dialysis membrane was placed between the two fungi (Zeilinger et al. 1999), but still

occurred when a cellophane membrane was used for this purpose (Cortés et al. 1998). This led to contradicting conclusions regarding the nature of the molecule triggering *ech42* gene expression (Cortés et al. 1998; Zeilinger et al. 1999); this issue was consequently solved by showing that the cellophane, but not the dialysis, membrane, was partially permeable to proteins of relatively large size (up to 100 kDa) (Kullnig et al. 2000). Thus the data from both studies (Cortés et al. 1998; Zeilinger et al. 1999) were in perfect agreement and showed that *ech42* is expressed before contact of *Trichoderma* with its host, probably representing one of the earliest event in mycoparasitism and biocontrol. By using two types of membranes (one permeable and one not permeable to proteins), which allowed the removal of either *Trichoderma* or *Rhizoctonia* colony from the plate and thus the performing of subsequent cultivations, Kullnig et al. (2000) also showed that a chitinase activity, secreted constitutively by *Trichoderma*, is essential for the triggering of *ech42* gene expression. The nature of this enzyme is still unknown; and it could very well be either (constitutive amounts of) the 42 kDa endochitinase itself or the 102 kDa protein of Inbar and Chet (1995), or any other constitutively formed chitinase or chitinases. To this end, Brunner et al. (2002) deleted the *nag1* (73-kDa *N*-acetyl- β -D-glucosaminidase encoding) gene of *T. atroviride*. These strains were unable to induce *ech42* gene transcription under conditions of carbon starvation or in the presence of fungal cell-walls, and also lacked the formation of other enzyme activities capable of hydrolyzing PNP-NAGlc, PNP-NAGlc₂, and PNP-NAGlc₃. Since the 102-kDa exochitinase does not occur in *T. atroviride* P1 (unpublished data), the 73-kDa enzyme may fulfil the role of the *T. harzianum* 102 kDa enzyme. Unfortunately, a characterization of the latter enzyme has not yet been published.

The obvious antifungal activity of *Trichoderma* chitinases has consequently lead to attempts to improve or alter biocontrol properties of strains by chitinase gene manipulations. Somewhat conflicting data have been reported on the effect of overexpression and/or deletion of selected chitinase genes of *Trichoderma*. Carsolio et al. (1999) found no difference between an *ech42*-disrupted strain and its parent *T. atroviride* IMI 206040 in the biocontrol activity in glasshouse tests against *Sclerotium rolfsii* and *R. solani* on cotton, and therefore concluded that *ech42* is not essential for biocontrol activity. In contrast, Woo et al. (1998); Baek et al. (1999) noted pronounced effects on the biocontrol efficacy of an *ech42* gene disruption mutant of *T. atroviride* P1 or *T. virens*, respectively. The latter authors reported an increased and decreased biocontrol activity against *R. solani* on cotton in strains of *T. virens* containing two *ech42* copies and a disrupted *ech42* gene copy. Woo et al. (1998) also observed a significant reduction in antifungal activity for the *ech42* disrupted strain and *in vivo* tests against *B. cinerea* by leaf inoculations of bean plants revealed a significant reduction of biocontrol ability of the disruptant strain. In contrast, a significant increase was noted for the biocontrol efficacy of soils heavily infested with *R. solani*. Macro- and

microscopic examinations of the attached seed coats suggested that the lack of the 42-kDa endochitinase may have stimulated the colonization of the spermo- and rhizosphere.

Other cell wall hydrolases, whose effect on biocontrol has been studied, are the chitinases *chit33* and *nag1* and the proteinase *prb1*. β -Glucanases have also been tried but their overexpression appears to be counteracted by overexpression of acid proteases (Delgado-Jarana et al. 2000). Using a constitutively expressed *pkil::chit33* fusion, Limón et al. (1999) obtained recombinant strains with higher antagonizing activity against *R. solani* on agar plates. However, results from experiments with these mutants performed in glasshouse or soil have not been reported. *T. harzianum* transformants carrying two to ten copies of the *prb1* gene significantly reduced the disease caused by *R. solani* in cotton plants under greenhouse conditions (Flores et al. 1997). Interestingly, culture filtrates of a *T. atroviride* *nag1*-delta strain, despite of their inability to induce chitinase gene expression (see earlier) exhibited a moderately reduced ability (40–50%) to protect beans against infections by *Rhizoctonia solani* and *S. sclerotiorum* (Brunner et al. 2003). Therefore, while *nag1* is essential for triggering chitinase gene expression in *T. atroviride*, the almost complete loss of chitinase activity only partially impairs biocontrol activity against *R. solani* and *S. sclerotiorum*. It is possible that this may be compensated by an increased formation of glucanolytic enzymes in this strain (unpublished data).

A more general approach towards improvement of the biocontrol properties of *T. harzianum* CECT 2413 was presented by Rey et al. (2001); they selected improved biocontrol mutants by testing for the ability to produce wider haloes on pustulan, a polymer of beta-1,6-glucan, as a carbon source. Interestingly, the mutants exhibited two- to four times more chitinase, beta-1,3- and beta-1,6-glucanase activities than the wild type, and produced about three times more extracellular proteins. This mutant performed better than the wild type during *in vitro* experiments, overgrowing and sporulating on *R. solani* earlier, killing this pathogen faster and exerting better protection on grapes against *B. cinerea*.

6.1 *Cis* and *Trans*-Acting Genetic Factors Relevant to the Expression of Biocontrol Genes

Lorito et al. (1996a) first used an *in vitro* approach to detect *cis*-acting motifs on the *ch42* promoter being involved in mycoparasitism. They confronted *Botrytis cinerea* on agar plates with *T. atroviride* P1, prepared crude protein extracts from mycelia harvested at different phases during mycoparasitism, and used them in electrophoretic mobility shift assays. Competition experiments, using oligonucleotides containing functional and nonfunctional consensus sites for binding of the carbon catabolite repressor Cre1 (5'-SYGGRG-3'; Kulmburg et al. 1993) provided evidence

that the complex from nonmycoparasitic mycelia involves the binding of Cre1 to both fragments of the *ech42* promoter. These findings are consistent with the presence of two and three consensus sites, respectively, for binding of Cre1 in the two *ech42* promoter fragments used. In contrast, the protein-DNA complex from mycoparasitic mycelia does not involve Cre1, as its formation is unaffected by the addition of the competing oligonucleotides. Based on these findings, they offered a preliminary model for regulation of *ech42* expression in *T. harzianum*, which subsequently involves: (a) binding of Cre1 to two single sites in the *ech42* promoter; (b) binding of a “mycoparasitic” protein/protein complex to the *ech42* promoter in vicinity of the Cre1 binding sites, and (c) functional inactivation of Cre1 upon mycoparasitic interaction to enable the formation of the “mycoparasitic” protein-DNA complex (Lorito et al. 1996a). The *cre1* gene from *T. harzianum* has been cloned (Ilmen et al. 1996), but no demonstration of its effect on biocontrol *in vivo* was as yet presented.

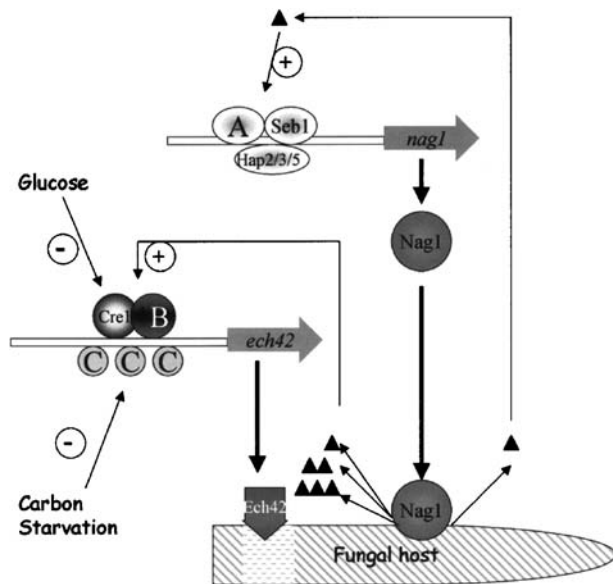


Figure 1 Scheme illustrating the hypothesis how chitinase gene expression could be triggered in *T. atroviride*, based on results from Brunner et al. (2002); Mach et al. (1999); Kullnig et al. (2000); Peterbauer et al. (2002a,b); Zeilinger et al. (1999). Circled plus and minus indicate activation and inactivation of a process, respectively, without implying the underlying mechanism. Proteins A, B and C refer to the Zn(6) cluster protein (Peterbauer et al. 2002a), the mycoparasitic regulator (Lorito et al. 1996a) and the BrIA-box binding starvation response repressor (see text), respectively. The black triangles indicate NAcGlc molecules, and symbolize NAcGlc, (NAcGlc)₂, and (NAcGlc)₃, respectively.

Another *cis*-acting element was recently identified that may contribute to the regulation of *ech42* gene expression: the *ech42* promoter sequence contains two short nucleotide sequences which resemble the consensus for binding of the *Aspergillus nidulans* brlA (bristle) regulator (5'-MRAGGGR-3'; Chang and Timberlake 1992). The encoded BrIA protein is a general regulator of conidial development, which itself responds to carbon starvation (Skromne et al. 1995). Cell-free extracts of *T. atroviride*, prepared from mycelia subjected to carbon starvation, form a specific, consensus-dependent complex with BrIA site-containing oligonucleotide fragments of the *ech42* promoter (K Brunner, CK Peterbauer, and CP Kubicek, unpublished data). Deletion of the promoter areas containing the BrIA sites *in vivo* resulted in a derepression of the starvation induced expression of *ech42*, but had no effect on the expression of *ech42* during sporulation. This motif therefore likely binds a new repressor of *Trichoderma* rather than a sporulation specific regulator.

The induction of *nag1* by chitin oligomers has been studied in more detail, using a combination of promoter deletion, *in vivo* footprinting, and EMSA experiments, proteins binding to an AGGGG-element, to a CCAGN₁₃-CTGG motif and to a CCAAT-box were identified (Peterbauer et al. 2002a,b). Disruption of either of the two former binding sites *in vivo* resulted in an almost complete reduction of induction of *nag1* expression by *N*-acetylglucosamine. The nature of the proteins binding to these three motifs is only partially understood: the spatial organization of the CCAGN₁₃CTGG motif would be compatible with the binding of a Zn(II)2Cys6-type zinc finger protein (Todd and Andrianopoulos 1997), whereas the CCAAT-box binds a protein complex consisting of at least three proteins Hap2, Hap3, and Hap5, which were originally described in *S. cerevisiae* and more recently characterized from *T. reesei* (Zeilinger et al. 2001). According to Narendja et al. (1999); Zeilinger et al. (2003), their function is the establishment of an open chromatin structure at the promoter.

The AGGGG-box is a motif which has been studied in detail in *Saccharomyces cerevisiae* and identified as a binding site for the Cys2His2 zinc finger proteins Msn2p and Msn4p, which are key regulators of the transcription of a number of genes coding for proteins with stress-protective functions (Ruis and Schüller 1995). In *Trichoderma*, the occurrence of the AGGGG-box is not restricted to the *nag1* promoter but also occurs in two other chitinase promoters, *ech42* and *chit33* (Lorito et al. 1996a; de las Mercedes Dana et al. 2001), consistent with a potential role in chitinase regulation. This would be compatible with a regulation of the expression of chitinase genes by metabolic stress, as shown both for *ech42* and *chit33* (see earlier). However, *nag1* is not upregulated by stress (CK Peterbauer, unpublished data), and the presence of this motif must therefore serve another function. In this context it is interesting to note that an AGGGG-motif was also identified in the cutinase promoter of *Haematonectria haematococca*, where it appeared to be involved in maintaining the basal expression level (Kämper et al. 1994). In *Yarrowia lipolytica*, the AGGGG motif is bound by

the Mhy1p protein, whose transcription is dramatically increased during the yeast-to-hypha transition (Hurtado and Rachubinski 1999).

In order to study whether a homologue of *S. cerevisiae* MSN2/4 and *H. haematococca* AGGGG-binding protein encoded by the open reading frame AAB04132 (which we call *sebl*, =stress element binding) has recently been cloned from *T. atroviride* (Peterbauer et al. 2002a,b). Its zinc finger domain has high amino acid sequence identity with *S. cerevisiae* Msn2/4 and the *H. haematococca* AGGGG-binding protein, and specifically recognizes the AGGGG sequence of the *ech42* and *nag1* promoter in band shift assays. However, a cDNA clone of *sebl* was unable to complement a MSN2/4 delta mutant of *S. cerevisiae*. Despite the presence of AGGGG elements in the promoter of the chitinase gene *nag1*, no differences in its expression were found between the parent and a *sebl*-delta-strain. The EMSA analyses with cell-free extracts of the *sebl*-delta still showed the presence of proteins binding to the AGGGG-element in *nag1* and *ech42*, and thus *sebl* does not encode the protein binding to this sequence in the chitinase promoters. Rather, *sebl* appears to be involved in osmotic stress response: *sebl*-mRNA accumulation was increased under conditions of osmotic stress (sorbitol, NaCl)—but not under other stress conditions (cadmium sulfate, pH, membrane perturbation), and growth of the delta-*sebl* strain was significantly more inhibited by the presence of 1M sorbitol and 1M NaCl than that of the wild-type strain (Peterbauer et al. 2002a,b).

7 CONCLUSIONS

This and the accompanying review (see chapter 57) show that the molecular biology of *Trichoderma* has made tremendous progress during the last decade, both from a methodical as well as theoretical perspective. Gene manipulation in *Trichoderma* is now routine, as are more sophisticated approaches to study gene expression and its regulation. In addition, working models such as the one developed in our laboratory on the induction of chitinases during mycoparasitism (Figure 1), can now be investigated and critically tested in more detail. Yet a drawback of the current situation is that so far mainly genes encoding extracellular enzymes have been studied, and even these mostly under laboratory conditions. Because of the redundancy of the genes encoding extracellular enzymes such as chitinases (cf. Baek et al. 1999), the role of the individual enzymes *in vivo* still needs to be critically assessed. Other factors may be more relevant in the field such as colonization (rhizosphere competence) and competition with the respective hosts. The development of strategies to clone the respective genes and their functional analysis will be the challenge of the future decade.

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The Biological Control Agent *Trichoderma* from Fundamentals to Applications

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1 INTRODUCTION

The practice of monoculture in modern agriculture enables us to continue to provide foodstuffs for the world's ever increasing population. Monoculture is, however, an ecologically unnatural situation, that is inherently unstable and offers considerable opportunity for the development of diseases. Plant disease control has now therefore become heavily dependent on fungicides to combat the wide variety of fungal diseases that threaten agricultural crops. Even with intensive fungicide use, the destruction of crop plants by fungal pathogens is a serious problem worldwide that annually leads to losses of about 15% (Logemann and Schell 1993). The use of pesticides in general, has also resulted in significant costs to public health and the environment. Studies aimed at replacing pesticides with environmentally safer methods are currently being conducted at many research centers. In this context, control of plant pests by the application of biological agents holds great promise as an alternative to the use of chemicals. It is generally recognized that biological control agents are safer and sounder environmentally than is reliance on the use of high volumes of fungicides and other antimicrobial treatments. The heightened scientific interest in biological control of plant pathogens is a response, in part, to growing public concerns over chemical pesticides. However, there is an equally greater need for biological control of pathogens that presently go uncontrolled or only partially controlled by these "traditional" means (Cook 1993). Thus, biological control should and can be justified on its own merits, without giving it importance at the expense of chemical controls.

Biocontrol must be effective, reliable, consistent, and economical before it becomes an important component of plant disease management. To meet these criteria, we must increase our understanding of the biology of the biocontrol agent in question, which in most cases is extremely limited. Furthermore, superior strains, together with delivery systems that enhance biocontrol activity, must be developed (Harman et al. 1989). In this context, many biological control agents can be modified genetically to enhance their attributes. In addition, we can now think of microorganisms with inhibitory activity against plant pathogens as potential sources of genes for disease resistance.

2 *TRICHODERMA* AS A BIOLOGICAL CONTROL AGENT

The potential for the use of *Trichoderma* species as biocontrol agents was suggested 70 years ago by Weindling (1932) who was the first to demonstrate the parasitic activity of members of this genus towards pathogens such as *Rhizoctonia solani* (Chet 1990; Weindling 1932). Since then, several species of *Trichoderma* have been tested as biocontrol agents; and have shown to attack a range of economically important aerial and soilborne plant pathogens (Chet 1987). In many experiments, showing successful biological control, the antagonistic *Trichoderma* was found to be a necrotrophic mycoparasite (Boosalis 1964; Chet and Elad 1982; Elad et al. 1983b). Mycoparasitism is defined as a direct attack on a fungal thallus, followed by nutrient utilization by the parasite (Chet et al. 1997). Necrotrophic mycoparasites, such as

Trichoderma, are those that kill the host cells before, or just after, invasion and use the nutrients released. These mycoparasites tend to be highly aggressive and destructive. They have a broad host range extending to wide taxonomic groups and are relatively unspecialized in their mode of parasitism. The antagonistic activity of necrotrophs is due to the production of antibiotics, toxins, or hydrolytic enzymes in such proportions as to cause death and destruction of their host (Manocha and Sahai 1993). In our view biocontrol by *Trichoderma* includes: (a) competition, (b) parasitism, (c) antibiosis, and (d) induction of defense responses in host plants, or the combination of some of them. Parasitism is a complex process including: (a) host recognition, (b) secretion of hydrolytic enzymes, (c) hyphae penetration and invasion (Figure 1), and (d) lysis of the host.

2.1 Host Recognition

In vitro, the first detectable interaction shows that the hyphae of the mycoparasite grow directly towards its host (Chet et al. 1981). This phenomenon appears as a chemotropic growth of *Trichoderma* in response to some stimuli produced by the host (Chet and Elad 1983). When the mycoparasite reaches the host, its hyphae often coil around it or attach to it by forming hooklike structures. Although not a frequent event production of appressoria at the tips of short branches has been observed. Coiling appears to be controlled by lectins present on the host hyphae. A *R. solani* lectin that binds to galactose and fucose residues was shown to agglutinate conidia of a mycoparasitic strain of *Trichoderma harzianum* but did not agglutinate two nonparasitic strains (Barak et al. 1985; Elad et al. 1983a).

D-glucose or D-mannose residues, apparently present on the cell walls of *T. harzianum*, inhibited the activity of a second lectin isolated from *Sclerotium rolfsii*. Inbar and Chet (1992; 1994) were able to mimic the fungus–fungus interaction *in vitro* using nylon fibers coated with either concanavalina A or the purified *S. rolfsii* lectin. During the interaction *Trichoderma* recognized and attached to the coated fibers, coiling around them and forming other mycoparasitism-related structures, such as appressoriumlike bodies and hyphal loops (Inbar and Chet 1992; 1994). Recently, using the biomimetic system we showed that different lectins induce coiling. Furthermore, coiling of *Trichoderma* around the fibers in the absence of lectins can be induced by applying cAMP or the heterotrimeric G protein activator mastoparan (Rocha-Ramírez et al. 2002). Transgenic lines that over-express the G α subunit coil at higher frequency than untransformed controls. Furthermore, transgenic lines that express an activated mutant protein with no GTPase activity coil at an even higher frequency. In addition, lines that express an antisense version of the gene do not appear to coil in the biomimetic assay (Rocha-Ramírez et al. 2002).

2.2 Host Invasion

It has been proposed that penetration of the host mycelium takes place by partial degradation of its cell wall (Elad et al. 1983b,c). Interaction sites have been stained by fluorescein isothiocyanate-conjugated lectins or calcofluor. The appearance of fluorescence indicated the presence of localized cell wall lysis at points of interaction between the antagonist and its host (Elad et al. 1983c). Furthermore,

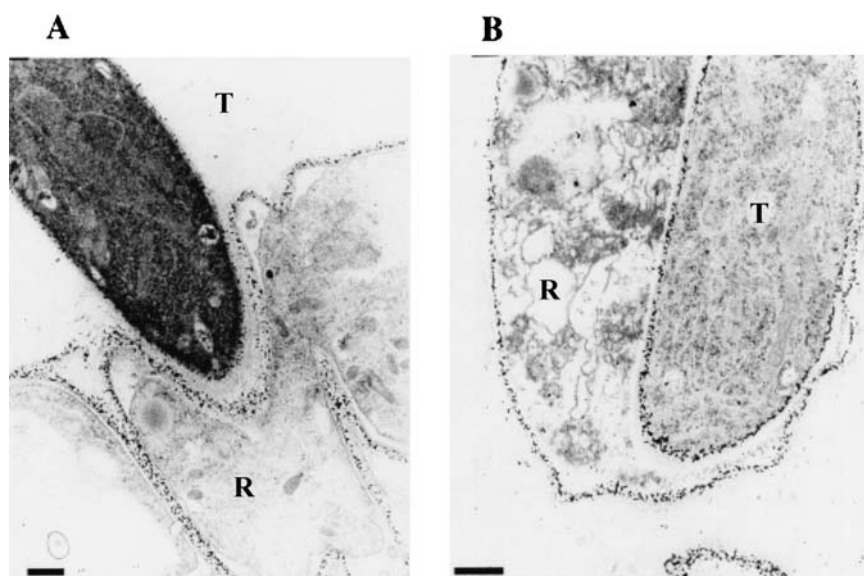


Figure 1 Transmission electron micrographs of *Trichoderma atroviride* parasiting *Rhizoctonia solani*. A) *T. atroviride* (T) penetrates *R. solani* (R). B) *Trichoderma* (T) grows inside an *R. solani* (R) hyphae.

analysis by electron microscopy has shown that during the interaction of *Trichoderma* spp. with either *S. rolfii* or *R. solani* the parasite hyphae contacted their host and perforated their cell walls. These observations led to the suggestion that *Trichoderma* produced and secreted mycolytic enzymes responsible for the partial degradation of the host's cell wall.

Indeed, *Trichoderma* produces a complex set of glucanases, chitinases, lipases, and proteases extracellularly when grown on cell walls of *R. solani* (Geremia et al. 1991; Vázquez-Garcidueñas et al. 1998). Table 1 summarizes the currently available information on this complex set of lytic enzymes produced by *Trichoderma*. Most attention has been paid to chitinases and several have been studied to some extent in different isolates or even species of the genus. The purification and characterization of three endochitinases secreted by *T. harzianum* was first reported by De la Cruz et al. (1992). They reported the isozymes to be 37, 33, and 42 kDa, respectively. Only the purified 42 kDa chitinase hydrolyzed *Botrytis cinerea* purified cell walls *in vitro*, but this effect was heightened in the presence of either of the other two isoenzymes (De la Cruz et al. 1992). The 42 kDa endochitinase has been found in most isolates. Recently, this enzyme has been proposed to play a major role in the regulatory circuits governing the expression of chitinases upon contact of *Trichoderma* with its host (Kubicek et al. 2001). Cloning of the genes coding for the 42 kDa secreted chitinases has allowed the construction of a phylogenetic tree,

which showed that they belong to family 18, class V of the glycosyl hydrolases. Interestingly, of the eight fungal species within this clade of the phylogenetic tree, all of them are either fungal or insect parasites and many of the corresponding genes have been implicated in their parasitic activity. However, the chitinolytic system of *Trichoderma* was recently found to be more complex. Two genes showing similarity to the one encoding the 33 kDa endochitinase described by De la Cruz et al. (1992) have been cloned from *T. virens* (Kim et al. 2002). These two genes are closely related, according to phylogenetic analysis, and belong to family 18, class III of the glycosyl hydrolases (Kim et al. 2002). Further, at least two types of *N*-acetyl- β -D-glucosaminidases belonging to family 20 of the glycosyl hydrolases have been identified in *T. harzianum* and *T. virens* (Draborg et al. 1995; Kim et al. 2002). Chit 36 is another antifungal chitinase recently isolated from *T. harzianum* TM. This 36 kDa protein shares no significant homology to either Chit33 or 42 (Viterbo et al. 2001). In addition, a 40 kDa chitobiosidase and a 28 kDa exochitinase have been purified (Deane et al. 1998; Harman et al. 1993).

In 1995, Haran and co-workers identified six distinct intracellular chitinases by activity on gels. This intracellular set of chitinases is apparently composed of two β -1,4-*N*-acetylglucosaminidases of 102 and 73 kDa, respectively, and four endochitinases of 52, 42, 33, and 31 kDa, respectively. From this set, the 102 kDa and the 73 kDa *N*-acetylglucosaminidases and the 42 kDa endochitinase, were

Table 1 *Trichoderma* genes encoding cell-wall degrading enzymes

Gene	<i>Trichoderma</i> spp.	Strain	Encoded protein	References
Th-En42	<i>T. atroviride</i>	P1	42-kDa endochitinase	Hayes et al. (1994)
ech42	<i>T. atroviride</i>	IMI206040	42-kDa endochitinase	Carsolio et al. (1994)
chit42	<i>T. harzianum</i>	CECT2413	42-kDa endochitinase	García et al. (1994)
ech1	<i>T. virens</i>	Tv29-8	42-kDa endochitinase	Kim et al. (2002)
th-ch	<i>T. harzianum</i>	Tam-61	42-kDa endochitinase	Fekete et al. (1996)
ENC1	<i>T. harzianum</i>	T25-1	42-kDa endochitinase	Draborg et al. (1996)
ech2	<i>T. virens</i>	Tv29-8	42-kDa endochitinase	Kim et al. (2002)
ech3	<i>T. virens</i>	Tv29-8	Endochitinase	Kim et al. (2002)
chit33	<i>T. harzianum</i>	CECT2413	33-kDa endochitinase	Limón et al. (1995)
cht1	<i>T. virens</i>	Tv29-8	33-kDa endochitinase	Kim et al. (2002)
cht2	<i>T. virens</i>	Tv29-8	33-kDa endochitinase	Kim et al. (2002)
chit36	<i>T. harzianum</i>	TM	36-kDa endochitinase	Viterbo et al. (2001)
nag1	<i>T. atroviride</i>	P1	73-kDa <i>N</i> -acetyl- β -D-glucosaminidase	Peterbauer et al. (1996)
exc1	<i>T. harzianum</i>	T25-1	73-kDa <i>N</i> -acetyl- β -D-glucosaminidase	Draborg et al. (1995)
nag1	<i>T. virens</i>	Tv29-8	<i>N</i> -acetyl- β -D-glucosaminidase	Kim et al. (2002)
exc2	<i>T. harzianum</i>	T25-1	<i>N</i> -acetyl- β -D-glucosaminidase ??	Draborg et al. (1995)
nag2	<i>T. virens</i>	Tv29-8	<i>N</i> -acetyl- β -D-glucosaminidase	Kim et al. (2002)
bgn13.1	<i>T. harzianum</i>	CECT 2413	78 kDa β -1,3-endoglucanase	De la Cruz et al. (1995)
bgn1	<i>T. virens</i>	Tv29-8	78 kDa β -1,3-endoglucanase	Kim et al. (2002)
bgn2	<i>T. virens</i>	Tv29-8	78 kDa β -1,3-endoglucanase	Kim et al. (2002)
gluc78	<i>T. atroviride</i>	P1	78 kDa exo- β -1,3-glucosidase	Donzelli et al. (2001)
bgn3	<i>T. virens</i>	Tv29-8	β -1,6-endoglucanase	Kim et al. (2002)
prb1	<i>T. atroviride</i>	IMI206040	31-kDa subtilisinlike protease	Geremia et al. (1993)

expressed differentially when *Trichoderma* was confronted with different hosts on plates (Haran et al. 1996; Inbar and Chet 1995). In conclusion, the complexity and diversity of the chitinolytic system of *T. harzianum* involves the complementary modes of action of a diversity of enzymes, all of which might be required for maximum efficiency against a broad spectrum of chitin containing plant pathogenic fungi.

Trichoderma atroviride also secretes β -1,3-glucanases in the presence of different glucose polymers and fungal cell walls. The level of β -1,3-glucanase activity secreted by *T. atroviride* was found to be proportional to the amount of glucan present in the inducer. The fungus produces at least seven extracellular β -1,3-glucanases upon induction with laminarin, a soluble β -1,3-glucan. The molecular weights of five of these enzymes fall in the range from 60 to 80 kDa, and their pIs are 5.0–6.8. In addition, a 35-kDa protein with a pI of 5.5 and a 39-kDa protein are also secreted (Vázquez-Garcidueñas et al. 1998). *bgn13*, which encodes a 78 kDa protein from *T. harzianum* was the first endoglucanase gene identified (De la Cruz et al. 1995). Recently, two genes showing high homology to *bgn13.1* have been identified in *T. virens*. These endoglucanases belong to family 55 of the glycosyl hydrolases (Kim et al. 2002). In addition, a 78 kDa exo- β -1,3-glucanase from *T. harzianum* has been characterized (Donzelli and Harman 2001).

From the set of glucanases produced by *Trichoderma* two β -1,6-endoglucanase genes have been identified, one in *T. harzianum* and one in *T. virens* (Kim et al. 2002; Lora et al. 1995). These two genes encode nearly identical proteins belonging to family 5 of the glycosyl hydrolases (Kim et al. 2002).

In 1993, Geremia and co-workers reported the isolation of a 31 kDa basic proteinase, which is secreted by *T. harzianum* during simulated mycoparasitism. The corresponding gene (*prb1*) was cloned and characterized (Geremia et al. 1993) and was the first report of the cloning of a mycoparasitism-related gene.

3 EXPRESSION OF MYCOPARASITISM RELATED GENES (MRGs)

Expression of extracellular chitinolytic enzymes is highly induced by growing *Trichoderma* on purified chitin, fungal cell walls, or mycelia as sole carbon source. It has been proposed that chitinolytic enzymes could be induced by soluble chito-oligomers (Reyes et al. 1989; St. Leger et al. 1986). This appears to be the case for the 73 kDa *N*-acetyl- β -D-glucosaminidase of *T. harzianum* and *T. atroviride*, which are induced not only by chito-oligomers but also by *N*-acetyl- β -D-glucosamine. The 42 kDa endochitinase of *T. harzianum* responds similarly to these compounds but *ech42* expression in *T. atroviride* is not induced by the products of chitin degradation (Carsolio et al. 1999; De la Cruz et al. 1993;

Mach et al. 1999; Schikler et al. 1998; Ulhoa and Peberdy 1991; Ulhoa and Peberdy 1993). Expression of *ech42* in *T. atroviride* is strongly induced during fungus–fungus interaction. Its expression is repressed by glucose, may be affected by other environmental factors, such as light and may even be developmentally regulated (Carsolio et al. 1994). In general, formation of most chitinolytic enzymes does not occur or is inhibited in the presence of glucose, sucrose, and chitinolytic end products (Carsolio et al. 1994; 1999; De la Cruz et al. 1993; García et al. 1994; Peterbauer et al. 1996; Ulhoa and Peberdy 1991). In addition, there is evidence suggesting that the expression of at least *ech42* of *T. atroviride* and *chit33* of *T. harzianum* is repressed by high levels of ammonium (Donzelli and Harman 2001; Mercedes de las et al. 2001). In this sense, the proteinase encoding gene *prb1* responds to carbon and nitrogen limitation. It has also been suggested that the MRGs *chit33*, *ech42*, and *prb1*, respond to other types of physiological stress (Mach et al. 1999; de las Mercedes et al. 2001; Olmedo-Monfil et al. 2002). Recently, we found that the response of *ech42* and *prb1* to nutrient limitation depends on the activation of conserved mitogen activated protein kinase (MAPK) pathways (Olmedo-Monfil et al. 2002).

The level of production of β -1,3-glucanases by *T. atroviride* is induced by the presence of cell walls of *M. rouxii*, *N. crassa*, *S. cerevisiae*, and *R. solani* (in ascending order of efficiency) and appears to be dependent on the amount of β -1,3-glucan present in the cell walls of these organisms (Vázquez-Garcidueñas et al. 1998). Additional results obtained with a filtrate of autoclaved *S. cerevisiae* cell walls suggest that the induction observed with cell walls may be triggered by two components, one extractable and one that remains cell-wall bound (Vázquez-Garcidueñas et al. 1998). In general, glucanase expression is repressed by glucose and in some cases, might be repressed by primary nitrogen sources (Donzelli and Harman 2001).

In summary, expression of all enzymes from the cell-wall degrading system of *Trichoderma* appears to be coordinated. Suggesting a regulatory mechanism involving substrate induction and catabolite repression. The expression of the system is controlled at the level of transcription as indicated by Northern analysis of the available genes (Carsolio et al. 1994; De la Cruz et al. 1995; Donzelli and Harman 2001; Flores et al. 1997; Geremia et al. 1993; Kim et al. 2002; Limón et al. 1995; Mercedes de las et al. 2001). An exciting finding in terms of signaling is that the induction of at least two MRGs, namely *prb1* and *ech42*, is triggered by a diffusible factor produced by the host (Cortés et al. 1998). Recently, it has been suggested that the activation of MRGs in response to the presence of the host, through such a molecule depends on the basal expression of *ech42* in *T. atroviride* (Kubicek et al. 2001). However, in *T. virens*, induction of four MRGs in response to cell walls in *ech1* (the homologue of *ech42*) knockout mutants is still observed. Whether a key molecule produced by the host *in vivo* switches on the expression of MRGs remains to be proven, as well as the role of *Ech42* in the production of such a molecule.

4 ANTIBIOSIS

The involvement of volatile and nonvolatile antibiotics in the antagonism by *Trichoderma* has been proposed (Dennis and Webster 1971a,b). Indeed some isolates of *Trichoderma* excrete growth inhibitory substances (Claydon et al. 1987; Ghisalberti and Sivasithamparam 1991; Sivan et al. 1984). Claydon et al. (1987) identified volatile alkyl pyrrols produced by *T. harzianum* that were inhibitory to a number of fungi *in vitro*. When these metabolites were added to a peat-soil mixture, they reduced the incidence of *R. solani*-induced damping-off on lettuce. However, there is insufficient evidence to be conclusive about their contribution to pathogen suppression and disease reduction *in situ*. *Trichoderma* also produces linear oligopeptides of 12–22 aminoacids (peptaibols), which are rich in α -aminoisobutyric acid, N-acetylated at the N-terminus and containing an amino alcohol at the C-terminus (Rebuffat et al. 1989; 1991). These oligopeptides are known to form voltage-gated ion channels in black lipid membranes and modify the membrane permeability of liposomes (El Hadji et al. 1989). This suggested a scenario where cell-wall degrading enzymes weaken the cell wall and peptaibol antibiotics inhibit synthesis of cell-wall components, impairing the capacity of the hyphae to repair the effect of cell-wall degrading enzymes (Lorito et al. 1996a,b). This hypothesis is supported by the fact that the action of cell-wall degrading enzymes is synergistic with that of antibiotics (Lorito et al. 1996a,b).

5 ROLE OF MRGs IN BIOCONTROL AND STRAIN IMPROVEMENT

A major challenge for researchers investigating the mechanisms involved in the parasitic activity of *Trichoderma* has been to establish the role of cell-wall degrading enzymes in the process. In fact, we have proposed to call all genes encoding cell-wall degrading enzymes MRGs, because of their apparent relation to the process, until their role is fully determined. Intensive efforts using genetic engineering are currently being directed at this goal. In 1997, Flores and coworkers generated transgenic *T. atroviride* lines carrying multiple copies of *prb1*. The resulting strains produced up to 20 times more proteinase and all strains tested were more effective in the control of *R. solani*. One strain reduced the disease incidence caused by *R. solani* on cotton plants to only 6% whereas the disease incidence for the nontransformed strain was 30% (Flores et al. 1997), demonstrating that *prb1* plays an important role in biocontrol and the feasibility of strain improvement through genetic engineering.

The role of the *Trichoderma* 42 kDa endochitinases in mycoparasitism has been addressed by genetic manipulation of the corresponding gene (*ech42* and *ech1*) in *T. atroviride* and *T. virens* (Baek et al. 1999; Carsolio et al. 1999; Woo et al. 1999). In *T. atroviride*, several transgenic strains carrying multiple copies of *ech42* were generated (Carsolio et al.

1999), as well as the corresponding gene disruptants (Carsolio et al. 1999; Woo et al. 1999). The level of extracellular endochitinase activity when *T. atroviride* was grown under inducing conditions increased up to 42 fold in multicopy strains as compared to the nontransformed strain. Multicopy transformants reduced disease incidence by about 10%. Furthermore, a 30% higher degradation of the chitin content in *R. solani* cell walls was observed during interaction with the overexpressing *Trichoderma* than with the wild type, when quantified by transmission electron microscopy (Carsolio et al. 1999). In the case of the gene disruptants no differences in their efficiency to control *R. solani* or *S. rolfii* were observed in greenhouse experiments, as compared to the nontransformed control strains (Carsolio et al. 1999). In a second study (Woo et al. 1999), a reduction of the antifungal activity *in vitro* of the *ech42* disrupted strains towards *B. cinerea* was observed. However, *in vivo* tests against *B. cinerea* by leaf inoculation of bean plants revealed a significant reduction of their biocontrol capacity. Contrasting with these results, *ech42* gene disruptants showed increased efficiency to control *R. solani* in soil. Similar experiments in *T. virens* showed increased and decreased biocontrol activity against *R. solani* on cotton using *ech1* overexpressing lines and gene disruptants, respectively (Baek et al. 1999).

The role of Chit33 and Chit36 from *T. harzianum* in biocontrol has also been tested by expressing the corresponding gene at high levels using the strong constitutive *pki* promoter (Limón et al. 1999; Viterbo et al. 2001). Test of *R. solani* control under greenhouse conditions suggested higher efficiency of *Trichoderma* transformants bearing the *chit36* gene under the *pki* promoter, but this did not reach statistical significance (Viterbo et al. 2001). The transgenic lines generated overexpressing Chit 33 showed higher antagonistic activity against *R. solani* on agar plates. However, *in vivo* experiments with these transgenic lines have not been reported.

In another attempt to increase the effectiveness of *T. harzianum*, it was transformed with a bacterial chitinase gene from *Serratia marcescens* under the control of the CaMV35S promoter. Two transformants showed increased constitutive chitinase activity and expressed a protein of the expected size (58 kDa). When evaluated in dual cultures against the phytopathogenic fungus *S. rolfii* both showed higher antagonistic activity, as compared to the nontransformed control (Haran et al. 1993). Unfortunately, no *in vivo* experiments were reported using these strains.

Recently, the role of glucanases in the interaction between *T. harzianum* and *Pythium ultimum* was studied (Benhamou and Chet 1997). Contact between the two fungi was accompanied by the deposition of a cellulose-enriched material at potential penetration sites. *Trichoderma* was able to penetrate this barrier, indicating that cellulolytic enzymes were produced. However, cellulase production was not the only critical trait involved in the process. A marked alteration of the β -1,3-glucan component of the *Pythium* cell wall was also observed, suggesting that β -1,3-glucanases played a key role in the antagonism. In yet another study,

T. longibrachiatum transformants carrying extra copies of the *egl1* gene (a cellulase encoding gene) were evaluated for their biocontrol activity against *P. ultimum* on cucumber seedlings (Migheli et al. 1998). The transformants showed a significantly higher level of expression of the *egl1* gene in comparison to the wild type under both inducing and noninducing growth conditions. Transformants with the *egl1* gene under the control of a constitutive promoter had the highest enzymatic activity. Both the endoglucanase activity and the transforming sequences were stable under non-selective conditions. When applied to cucumber seeds sown in *P. ultimum*-infested soil, *T. longibrachiatum* transformants with increased inducible or constitutive *egl1* expression generally were more suppressive than the wild-type strain.

Biocontrol agents tolerant to specific pesticides could be constructed using molecular techniques. Resistance to the fungicide benomyl is conferred by a single amino-acid substitution in one of the β -tubulins of *T. viride*; the corresponding gene has been cloned and proven to work in other *Trichoderma* species (Goldman et al. 1993), thereby producing a biological control agent that could be applied simultaneously or in alternation with the fungicide. However, these strains have not been tested in biocontrol experiments. Molecular techniques may eventually be used to transfer several beneficial traits, such as the production of one or more antibiotics and pesticide tolerance, to an aggressive phyllosphere colonizer.

6 COMPETITION

Competition occurs between microorganisms when space or nutrients (i.e., carbon, nitrogen, and iron) are limiting and its role in the biocontrol of plant pathogens has been studied for many years, with special emphasis on bacterial biocontrol agents (Weller 1988). Implicit in this definition is the understanding that combative interactions such as antibiotic production, mycoparasitism, or the occurrence of induced resistance in the host are excluded even though these mechanisms may form an important part of the overall processes occurring in the interaction. In the rhizosphere, competition for space as well as nutrients is of major importance. Thus, an important attribute of a successful rhizosphere biocontrol agent would be the ability to remain at high population density on the root surface providing protection of the whole root for the duration of its life. Recently, it was found that a strain of *T. harzianum* (T-35) that controls *Fusarium* spp. on various crops might take advantage of competition for nutrients and rhizosphere colonization (Sivan and Chet 1989).

7 INDUCED RESISTANCE

Induced resistance is a plant response to challenge by microorganisms or abiotic agents such that following the inducing challenge *de novo* resistance to pathogens is shown

in normally susceptible plants (de Wit 1985). Induced resistance can be localized, when it is detected only in the area immediately adjacent to the inducing factor or systemic, where resistance occurs subsequently at sites throughout the plant. Both localized and systemically induced resistances are nonspecific.

Recently, the potential of *T. harzianum* T-203 to trigger plant defense responses was investigated by inoculating roots of cucumber seedlings with *Trichoderma* in an aseptic, hydroponic system (Yedidia et al. 1999). *Trichoderma*-treated plants were more developed than nontreated plants throughout the experiment. Electron microscopy of ultrathin sections from *Trichoderma*-treated roots revealed penetration of *T. harzianum*, mainly to the epidermis and outer cortex. Strengthening of the epidermal and cortical cell walls was observed, as well as deposition of newly formed barriers. These typical host reactions were found beyond the sites of potential fungal penetration. Wall appositions contained large amounts of callose and infiltrations of cellulose. Further biochemical analyses revealed that inoculation with the fungus resulted in increased peroxidase and chitinase activities in roots and leaves of treated seedlings, providing evidence that *T. harzianum* may induce systemic resistance mechanisms in cucumber plants (Yedidia et al. 1999).

8 PLANT GROWTH PROMOTION

Microbial interactions with plant roots are known to affect profoundly plant nutrient status and, for manganese at least, to affect plant resistance to pathogens (Huber and McCay-Buis 1993). In addition to their biocontrol characteristics, *Trichoderma* species also exhibit plant-growth-promoting activity (Baker 1989; Chet 1987; Harman and Bjorkman 1998; Inbar et al. 1994; Kleifeld and Chet 1992; Naseby et al. 2000). In spite of their theoretical and practical importance, the mechanisms responsible for the growth response due to *Trichoderma* have not been investigated extensively. Since growth enhancement has been observed in the absence of any detectable disease (Chang et al. 1986; Harman and Bjorkman 1998; Naseby et al. 2000) and in sterile soil (Windham et al. 1986), it is not thought to be a side effect of suppression of disease or minor plant pathogens. Other mechanisms, including production of hormonelike metabolites and release of nutrients from soil or organic matter, have been proposed (Kleifeld and Chet 1992; Windham et al. 1986).

The plant-growth-promoting capacity of *T. harzianum* to solubilize *in vitro* some insoluble or sparingly soluble minerals via three possible mechanisms: acidification of the medium, production of chelating metabolites, and redox activity was recently investigated (Altamore et al. 1999). *T. harzianum* was able to solubilize MnO_2 , metallic zinc, and rock phosphate (mostly calcium phosphate). Fe_2O_3 , MnO_2 , Zn, and rock phosphate were also solubilized by cell-free culture filtrates. A size exclusion chromatographic separation of the components of the culture filtrates indicated the presence of a complexed form of Fe but no chelation of Mn.

In liquid culture, *T. harzianum* also produced diffusible metabolites capable of reducing Fe(III) and Cu(II). Solubilization of metal oxides by *Trichoderma* involves both chelation and reduction (Altamore et al. 1999).

9 TRICHODERMA AS A SOURCE OF GENES FOR CROP IMPROVEMENT

One of the major goals of plant genetic engineering is to protect plants from diseases. There are many examples of the introduction of chitinase genes into plants resulting in an enhancement of resistance of the host plant to fungal pathogens (Broglie et al. 1993; Lin et al. 1995; Vierheilig et al. 1993). However, the desired levels of resistance for successful commercial application have not yet been reached. The use of potent chitinases with proven antifungal activity is thus an attractive alternative. Because Ech42 from *T. atroviride* fulfills these criteria, the corresponding gene was introduced into tobacco and potato (Lorito et al. 1998). High expression levels of the fungal gene were obtained in different plant tissues, with no visible effect on plant growth and development. Substantial differences in endochitinase activity were detected among different transformed lines. Transgenic lines were highly tolerant or completely resistant to the foliar pathogens *Alternaria alternata*, *Alternaria solani*, *B. cinerea*, and the soilborne pathogen *R. solani*. Interestingly, the levels of tolerance reached in these experiments were higher than those previously achieved by expression of bacterial or plant chitinases (Lorito et al. 1998).

A similar strategy was used to improve scab resistance of apple (Bolar et al. 2000). The endochitinase gene (*ech42*), as cDNA and genomic clones, was transferred into apple cv. Marshall McIntosh. Eight lines propagated as grafted and self-rooted plants were inoculated with *Venturia inaequalis*. Six transgenic lines expressing the endochitinase were more resistant than controls. Disease severity in the transgenic lines tested compared with nontransformed controls was reduced, as well as the number of lesions and the leaf area infected. However, in contrast with the results previously reported (Lorito et al. 1998), expression of the endochitinase also had negative effects on the growth of both inoculated and uninoculated plants (Bolar et al. 2000). In a more recent investigation the same group introduced either an endochitinase or an exochitinase, both from *Trichoderma*, into apple plants (Bolar et al. 2001). In agreement with their previous results resistance to *V. inaequalis* correlated with the level of expression of either enzyme. Plants expressing both enzymes simultaneously were more tolerant than plants expressing either enzyme alone. Their results indicate that the two enzymes acted synergistically to limit disease development.

10 CONCLUSIONS

Although *Trichoderma* is widely used in the field to control plant diseases and its commercialization has significantly

increased, our understanding of the mechanisms used by *Trichoderma* to antagonize phytopathogenic fungi is still very limited.

Little is known on the signaling pathways that determine host recognition, although there is evidence of the involvement of conserved signaling pathways such as heterotrimeric G proteins in hyphal coiling. At later stages of the interaction, MAPK pathways appear to participate in the regulation of the expression of MRGs. However, we are still just beginning to untangle the networks determining host recognition.

An important number of genes encoding cell-wall degrading enzymes have been cloned. In most cases, their expression correlates with conditions that simulate the actual interaction with the host and some of them have even been used to generate improved strains. However, the fact that several of the cloned MRGs respond to multiple environmental signals that they are subjected to catabolite repression, and that none of them is expressed specifically at the sites of interaction, suggests that these genes maybe part of a specialized saprophytic response. Thus, the corresponding enzymes are more likely to participate in the utilization of the host's cellular components as a food supply at the end of the parasitic process. An alternative explanation is that the interaction of *Trichoderma* with a host is interpreted by the parasite as a stress signal and that MRGs are in fact stress responsive genes. It is likely that genes coding for key enzymes such as those expressed specifically at the site of interaction where penetration or cell wall perforations are observed, have not been yet identified. The use of functional genomics strategies will certainly be a major step towards the identification of genes playing key roles in mycoparasitism by *Trichoderma*. *Trichoderma* has already proven to be an important source of genes for engineering plants for pathogen resistance. Yet, there is still a complete battery of genes that should be tested for this purpose, as well as combinatory strategies using several *Trichoderma* genes. Induction of defense responses in host plants and plant growth promotion are important attributes of *Trichoderma*, whose study was neglected for a long time. The recent evidence on these two aspects makes *Trichoderma* an even more attractive organism for large-scale application as a biological control agent. In spite of our limited knowledge on the mechanisms underlying the mycoparasitic activity of *Trichoderma*, it is clear that it is an excellent model system for the study of interfungal parasitic relationships and that it has an enormous potential for a variety of biotechnological applications.

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Biological Control of Fungal Diseases on Vegetable Crops with Fungi and Yeasts

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1 INTRODUCTION

Vegetable crops may be produced as both fresh market and processed commodities and can be grown under field conditions or in controlled environments, such as glasshouses or other similar structures. There are numerous fungal diseases that attack a wide range of these vegetable crops (Howard et al. 1994), thereby reducing crop yield and quality. Methods for disease control have included the use of cultural practices to reduce pathogen inoculum and disease incidence, development of resistant cultivars, as well as the application of chemical fungicides to inhibit pathogen development. The use of biological control strategies has also demonstrated the potential of fungi and yeasts in reducing a range of fungal pathogens that cause various diseases on vegetable crops.

In this chapter, some examples of recent successes in biological control of fungal diseases of vegetable crops using fungi and yeasts, and the mechanisms by which pathogen control was achieved will be reviewed. In addition, the utilization of techniques in biotechnology to aid in the implementation of biological control strategies for disease control will be reviewed. These include techniques to investigate mechanism(s) of action of the biological control agent, development of strains with enhanced efficacy through genetic manipulation, monitoring the growth and spread of biocontrol agents using molecular techniques, and characterization of strains using genetic markers and biochemical methods.

The diseases to be considered in this chapter for which biocontrol strategies have been described include those caused by pathogenic fungi that infect the seed and early

stages of seedling growth, causing seed decay and damping-off. Examples of these fungi are *Rhizoctonia solani* Kühn, various species of *Fusarium* and *Pythium*, and *Sclerotium rolfsii* Sacc. Fungal pathogens that infect the roots and crown of developing plants, causing root and crown rots and vascular wilts, have also been researched for biological control strategies. These include fungi such as *Pythium* spp., *Fusarium* spp., and *Sclerotinia sclerotiorum* (Lib.) de Bary. A third group of foliar-infecting fungi of vegetable crops that cause leaf spots and blights and stem infection, also have biological control strategies developed against them. These include *Botrytis cinerea* Pers. ex Fr. (gray mold), *Didymella bryoniae* (Auersw.) Rhem (gummy stem blight), *S. sclerotiorum* (white mold), and *Sphaerotheca* and *Erysiphe* spp. (powdery mildews).

Many different fungal and yeast biological control agents have been identified and evaluated for disease control potential against the above-mentioned pathogens, and some have been formulated and brought to market to provide disease control options for producers of vegetable crops. The use of biological control agents may be particularly attractive for vegetable crops grown in glasshouses, due to the high market value of these crops and the possibility for control of environmental parameters, particularly temperature and relative humidity (Paulitz and Bélanger 2001). These are important variables that can significantly influence the efficacy of biological control agents under natural field conditions (Paulitz 1997). The rationale for development of biological control agents against fungal diseases on vegetable crops was to provide an additional/alternative approach to augment/replace the use of

chemical fungicides, to provide a level of disease control in the absence of crop genetic resistance, and to augment cultural control practices to further minimize the impact of these diseases and reduce chemical residues in food. For example, chemical fungicides typically have provided adequate control of many fungal pathogens. However, fungicide resistance problems, concerns regarding pesticide residues, and revocation of registration of certain widely-used fungicides, have led to increased activity in the development of biocontrol agents against foliar fungal pathogens.

For a potential biological control agent to reach the stage of commercial deployment, numerous criteria have to be satisfied and considerable data need to be obtained to demonstrate aspects of efficacy, survival, adaptability, and scale-up. These aspects are reviewed elsewhere (Avis et al. 2001a; Cook 1993; Harman 2000; Lumsden et al. 1996) and will not be discussed in this chapter. Several agricultural chemical companies and a number of companies with specialized agricultural products have invested in the discovery and development of biological control agents to complement synthetic pesticides for the control of diseases on horticultural crops. These products are targeted to markets where they have the best chance of performing and where there is the most need, e.g., for control of seed and root-infecting pathogens on seedlings (Whipps 2001). A range of commercially available biological control products for plant disease control is now available (Fravel 2000) and are more likely to be brought to market in the future. Molecular methods have been described that can be adapted for use to ensure quality control and monitoring of the biocontrol agents (Avis et al. 2001a).

2 BIOCONTROL OF SEED ROTS AND DAMPING-OFF DISEASES

Germination of plant seeds is accompanied by the exudation of host nutrients into the soil environment, which frequently attract potentially damaging fungi such as *Pythium* spp., *R. solani*, and *Fusarium* spp. These fungi utilize the seed and root exudates as an energy source for germination and growth, and subsequently penetrate and colonize the seed and root hairs, causing rot and damping-off of emerging seedlings. These fungi are favored by cool (15–20°C) and moist conditions. Fungal biological control agents have been described which when applied to the surface of seed, to the planting substrate, or when applied shortly after seed germination, can utilize the host nutrient exudates and colonize the seed and developing roots to compete with and exclude the pathogenic fungi. In addition, many of these biocontrol agents secrete hydrolytic enzymes and antibiotics that inhibit the development of the pathogenic fungi. The most widely-researched of these biocontrol agents are species of *Trichoderma* and *Gliocladium* and to a lesser extent *Penicillium* spp. (Table 1).

3 BIOCONTROL OF ROOT AND CROWN ROTS AND VASCULAR WILT DISEASES

Pathogenic fungi which infect the root system and crown tissues through root hairs, natural openings, or wounds, can rapidly colonize these tissues and enter the vascular tissues, causing decay and death of the plants. Most of these pathogens infect during the early stages of plant development, although disease symptoms may only be manifested later. Fungal biological control agents have been described which when applied to the seed, planting medium, or roots of plants, can colonize the root system, occupy potential infection sites, and compete with the pathogen. In addition, these agents may enhance resistance in the plants through induction of various defense responses, and secrete hydrolytic enzymes and antibiotics that inhibit pathogen growth and development. The most widely-researched of these biocontrol agents are antagonistic fungi (*Trichoderma* and *Gliocladium* species) and nonpathogenic, closely-related fungi (*Pythium*, *Fusarium*, and *Rhizoctonia* species), as well as mycoparasitic distantly related fungi such as *Talaromyces*, *Coniothyrium*, *Sporidesmium*, *Stachybotrys*, and *Verticillium* (Table 1).

3.1 *Trichoderma* As a Biocontrol Agent of Seedling Root, Crown, and Vascular Diseases

3.1.1 *Trichoderma* Species Identification

The genus *Trichoderma* contains species that occur in soils throughout the world. Most species are fast-growing saprophytes with the ability to survive under a range of environmental conditions by utilizing different substrates for growth (Hjeljord and Tronsmo 1998; Samuels 1996). The most common biological control agents in the genus *Trichoderma* have been reported to be strains of *Trichoderma virens*, *T. harzianum*, and *T. viride* (Hermosa et al. 2000). Characterization of 16 biocontrol strains, identified previously as *Trichoderma harzianum* Rifai and one biocontrol strain recognized as *T. viride*, has been carried out using several molecular techniques. A certain degree of polymorphism was detected among isolates in hybridizations using a probe of mitochondrial DNA. Sequencing of internal transcribed spacers 1 and 2 (ITS1 and ITS2) of ribosomal DNA revealed three different ITS lengths and four different sequence types. Phylogenetic analysis based on ITS1 sequences, including type strains of different species, clustered the 17 biocontrol strains into four groups: *T. harzianum*–*T. inhamatum* complex, *T. longibrachiatum*, *T. asperellum*, and *T. atroviride*–*T. koningii* complex. ITS2 sequences were also useful for locating the biocontrol strains in *T. atroviride* within the complex *T. atroviride*–*T. koningii*. None of the biocontrol strains studied corresponded to biotypes Th2 or Th4 of *T. harzianum* that cause mushroom green mold. A similar study by Dodd et al. (2000) utilized ITS1 and ITS2 sequence data to group 50 isolates of *Trichoderma* species with biocontrol potential, while ITS1

Table 1 Examples of fungal biological control agents that prevent seed rots, damping-off, and root, crown, and vascular diseases caused by pathogenic fungi on vegetable crops

Biocontrol agent	Target pathogen and host	References
<i>T. harzianum</i>	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i> on tomato	Datnoff et al. (1995), Nemec et al. (1996), and Sivan and Chet (1993)
<i>T. harzianum</i> , <i>T. hamatum</i>	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> on tomato	Larkin and Fravel (1998)
<i>T. harzianum</i>	<i>P. capsici</i> on pepper	Ahmed et al. (1999)
<i>T. harzianum</i>	<i>P. ultimum</i> and <i>R. solani</i> on bean	Woo et al. (1999)
<i>T. hamatum</i> , <i>T. harzianum</i> , <i>T. viride</i> , <i>T. virens</i>	<i>R. solani</i> on eggplant	Lewis et al. (1998)
<i>T. harzianum</i>	<i>S. sclerotiorum</i> on pea	Knudsen and Eschen (1991)
<i>T. longibrachiatum</i>	<i>P. ultimum</i> on cucumber	Migheli et al. (1998)
<i>G. virens</i>	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> on tomato	Larkin and Fravel (1998)
<i>G. virens</i>	<i>P. ultimum</i> on cucumber; <i>R. solani</i> on peas	Koch (1999)
<i>G. virens</i> GL-3	<i>R. solani</i> , <i>P. ultimum</i> , <i>S. rolfisii</i> , and <i>F. oxysporum</i> on tomato and pepper	Mao et al. (1998)
<i>G. virens</i> GL-3, GL-21	<i>S. rolfisii</i> on carrot	Ristaino et al. (1994)
<i>G. catenulatum</i> J1446	<i>Pythium</i> on cucumber	Niemi and Lahdenperä (2000) and Punja and Yip (unpublished)
<i>P. oxalicum</i>	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> on tomato	De Cal et al. (1999)
Nonpathogenic <i>F. oxysporum</i>	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> on tomato	Alabouvette et al. (1993), Duijff et al. (1998), and Fuchs et al. (1999)
Nonpathogenic <i>F. oxysporum</i> , <i>F. solani</i>	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> on tomato	Larkin and Fravel (1998)
Nonpathogenic <i>F. oxysporum</i>	<i>F. oxysporum</i> f. sp. <i>cucumerinum</i> on cucumber	Mandeel and Baker (1991)
Nonpathogenic <i>R. solani</i>	<i>R. solani</i> and <i>Pythium</i> on cucumber and pepper	Cubeta and Echandi (1991), Harris and Adkins (1999), and Villajuan-Abgona et al. (1996)
Nonpathogenic <i>Rhizoctonia</i>	<i>R. solani</i> on bean, cabbage	Jabaji-Hare et al. (1999) and Ross et al. (1998)
<i>P. oligandrum</i>	<i>Pythium</i> spp. on cress	McQuilken et al. (1992)
<i>P. oligandrum</i>	<i>V. dahliae</i> on pepper	Al-Rawahi and Hancock (1998)
<i>T. flavus</i>	<i>S. rolfisii</i> on bean	Madi et al. (1997)
<i>T. flavus</i>	<i>V. dahliae</i> on eggplant	Engelkes et al. (1977) and Stosz et al. (1996)
<i>T. flavus</i>	<i>S. rolfisii</i> on bean	Madi et al. (1997)
<i>C. minitans</i>	<i>S. sclerotiorum</i> on lettuce	Budge and Whipps (2001)
<i>S. sclerotivorum</i>	<i>S. minor</i> on lettuce	Adams and Fravel (1990)
<i>C. foecundissimum</i>	<i>P. ultimum</i> and <i>R. solani</i> on eggplant and pepper	Lewis and Larkin (1998)

sequence data and RFLP analysis were used to distinguish amongst isolates of *T. harzianum* (Gams and Meyer 1998). These studies demonstrated the utility of molecular methods to resolve the identity of strains of *Trichoderma* with potential biocontrol activity that were overlapping in morphological features. This approach could also be used to develop strain-specific markers for a desired biocontrol strain. Molecular markers were developed and used to detect and trace a strain of *T. hamatum* in potting mix (Abbasi et al. 1999).

3.1.2 *Trichoderma* Biocontrol Activity

Several studies have shown that *T. harzianum* can control diseases caused by many root-infecting pathogens, including *Fusarium*, *Rhizoctonia*, and *Pythium* (Table 1). *T. harzianum* strain KRL-AG2, commercially formulated as F-Stop, when added to a potting soil mix prior to seeding with tomatoes, reduced the incidence and severity of *Fusarium* root and crown rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Datnoff et al. 1995). *T. harzianum* strain T-22 also provided control of *Fusarium* crown rot of tomato (Nemec et al. 1996), and the fungus could be recovered from the roots of treated plants 26 days after application, suggesting it had colonized the roots. *T. hamatum* reduced the incidence of *Fusarium* wilt of tomato, caused by *F. oxysporum* f. sp. *lycopersici*, when added to potting medium prior to seeding (Larkin and Fravel 1998). A commercial formulation of *T. harzianum* (Rootshield strain T-22) was also evaluated against this disease and was found to significantly reduce it when incorporated into potting mix at 0.2% (Larkin and Fravel 1998). Strain T-22 of *T. harzianum* was generated by fusing a mutant strain capable of colonizing plant roots with a strain able to compete with bacteria under iron-limiting conditions using protoplast fusion techniques (Harman 2000). This new strain had the enhanced ability to colonize the root system of host plants, resulting in greater efficacy as a biological control agent for long-term root protection (Harman 2000; Harman and Björkman 1998; Sivan and Harman 1991).

A number of commercial formulations are available that contain strains of *T. harzianum* for use against different diseases on a wide range of crops. These products are registered for use against a number of soilborne pathogens. Among these products, RootShield™, T-22G, and T-22 Planter Box™ contain *T. harzianum* strain T-22 that is able to survive well in the rhizosphere of plants (Fravel 2000).

3.1.3 *Trichoderma* Mechanism of Action

Trichoderma species can confer biological control against soilborne diseases through a number of mechanisms, including antibiosis, parasitism, competition, and the induction of host plant resistance (Hjeljord and Tronsmo 1998). *Trichoderma* species are known to produce a range of volatile and nonvolatile secondary metabolites, some of which inhibit other microorganisms and are considered to be antibiotics. These fungi can also penetrate and infect

pathogen structures, such as hyphae, causing them to be degraded through the production of cell wall degrading enzymes, such as chitinases, glucanases, cellulases, and proteinases (Geremia et al. 1993; Schirmbock et al. 1994; Thrane et al. 1997; Zeilinger et al. 1999). *Trichoderma* species can compete with pathogens for nutrients, rapidly colonize a substrate and exclude pathogens from infection sites, and colonize senescing tissues and wounds to reduce pathogen colonization (Hjeljord and Tronsmo 1998). Some strains of *Trichoderma* are good root colonizers (rhizosphere competent). It has also been reported that *T. harzianum* (strain T-22) has the ability to directly enhance root growth and plant development in the absence of pathogens (Harman 2000), and it has been suggested that this was due to the production of a growth-regulating factor by the fungus (Windham et al. 1986). Altomare et al. (1999) proposed that the ability of *T. harzianum* to increase plant growth was partially due to the organism's ability to solubilize nutrients, thus making them more available to host plants. These observations indicate the versatility through which *Trichoderma* species can manifest biological control activity. Finally, it has been reported that a strain of *T. harzianum* was able to trigger host defense mechanisms in cucumber plants through enhanced cell wall depositions and induction of defense enzymes, suggesting an indirect effect in the host plant by the biocontrol agent (Yedidia et al. 1999).

3.1.4 Biotechnological Manipulations of *Trichoderma*

Techniques in biotechnology have been applied to elucidate the role of hydrolytic enzymes, such as chitinases and glucanases, in mycoparasitism by *Trichoderma* that could lead to biological control activity. Transformants of *T. longibrachiatum* expressing extra copies of the β -1,4-endoglucanase gene *eglI* were found to be better at suppressing *Pythium* development on cucumber compared to wild-type strains (Migheli et al. 1998). In addition, transformants of *T. harzianum* overproducing the proteinase gene *prb1* had up to a five-fold increase in ability to protect cotton seedlings from *R. solani* (Flores et al. 1996). Transformants of *T. harzianum* overexpressing an endochitinase gene *chit33* were more effective in inhibiting growth of the pathogen *R. solani* *in vitro* compared with wild-type strains (Limón et al. 1999). A mutant of *T. harzianum* that was selected for its enhanced ability to hydrolyze pustulan, a polymer of β -1,6-glucan, had 2–4 times more chitinase, β -1,3 and β -1,6 glucanase activity compared to the wild-type, produced three times more extracellular proteins and other compounds, and showed greater inhibition of *B. cinerea* *in vitro* (Rey et al. 2001). These studies reaffirm the roles played by fungal enzymes in biocontrol of plant pathogens and also highlight the successes in manipulating biocontrol strains to genetically engineer them to enhance efficacy. Specificity in the activity of the hydrolytic enzymes was suggested in a study by Woo et al. (1999), in which the endochitinase *ech42* gene encoding for the secreted 42 Kda endochitinase

(CHIT 42) was silenced by targeted disruption; it was found that the endochitinase-deficient mutant had similar activity as the wild-type strain against *Pythium ultimum*, but had enhanced activity against *R. solani*, and reduced activity against *B. cinerea*.

A genetically marked strain of *T. harzianum* was developed by transformation with the β -glucuronidase (*uid A*) gene and the hygromycin B (*hygB*) gene for use in population dynamics studies (Thrane et al. 1995). Techniques utilizing protoplast transformation as well as particle bombardment of conidia have been described for *Trichoderma* (Lorito et al. 1993; Thrane et al. 1995). Population densities of the transformed strain could be monitored in a potting mix (Green and Jensen 1995), and the presence of the biocontrol agent around wounded tissues was reported.

3.2 *Gliocladium* As a Biocontrol Agent of Seedling, Root, Crown, and Vascular Wilt Diseases

Gliocladium virens Miller, Giddens and Foster, a biocontrol agent of a wide range of fungal pathogens, is now classified in the genus *Trichoderma* due to similar morphological characteristics that are shared with members of this genus and DNA analysis supported the inclusion of *G. virens* with the *Trichoderma* genus (Rehner and Samuels 1994). One characteristic of *G. virens* is the ability to produce the antibiotic metabolites gliotoxin and viridin, a characteristic not generally shared with other species of *Trichoderma* (Papavizas 1985). *G. virens* (*T. virens*) strain GL-3 was evaluated as a seed treatment on tomato against several pathogens, including *R. solani*, *P. ultimum*, *S. rolfii*, and *F. oxysporum* f. sp. *lycopersici*. The treatment resulted in significantly higher seedling establishment (Mao et al. 1998).

A commercial formulation of strain GL-21 of *G. virens* is registered for use in the United States, under the trade name SoilGard™, and can control *P. ultimum* and *R. solani* on vegetables and ornamental seedlings (Koch 1999; Lumsden et al. 1996). Like *T. harzianum* strain T-22, certain strains of *G. virens* have the ability to colonize the rhizosphere of plant roots (Harman 2000). In addition, the production of gliotoxin occurs rapidly (within a few hours) and can persist for several days to provide high levels of pathogen suppression (Lumsden et al. 1992; Wilhite and Straney 1996). Gliotoxin has also been shown to act synergistically with endochitinase in *G. virens* (Di Pietro et al. 1993).

A related species, *G. catenulatum* Gilman and E. Abbot, has been reported to be effective in reducing the incidence of damping-off diseases, caused by *P. ultimum* and *R. solani* (McQuilken et al. 2001). Incorporation of a wettable powder formulation of *G. catenulatum* strain J1446 into peat-based growing media or application as a drench reduced damping-off due to *P. ultimum* and *R. solani*. Two commercial formulations of *G. catenulatum* strain J1446 (Prestop and Primastop) have been recently developed (Fravel 2000; Niemi

and Lahdenperä 2000). Primastop is currently registered in Europe and in a number of regions of the United States, and Prestop is expected to be registered in the near future (Niemi and Lahdenperä 2000). *G. catenulatum* strain J1446 was able to colonize cucumber roots extensively 5 weeks following its application, indicating that the fungus has the ability to survive and proliferate in the rhizosphere of plants. This rhizosphere competence, coupled with its reported ability to act as a mycoparasite (McQuilken et al. 2001) makes *G. catenulatum* a strong candidate as a biological control agent against a number of vegetable diseases. We have evaluated this biocontrol agent against *Pythium* root and crown rot of cucumber caused by *P. aphanidermatum*. Application at the time of seeding significantly reduced plant mortality and enhanced seedling growth (Figure 1).

3.3 Nonpathogenic Fungi As Biocontrol Agents of Seedling, Root, Crown, and Vascular Wilt Diseases

Nonpathogenic and hypovirulent strains of fungi that are closely related taxonomically to plant pathogenic species have been reported to provide biological control of a number of pathogens, including species of *Fusarium*, *Rhizoctonia*, and *Pythium*. Competition by nonpathogenic strains for host plant nutrients, colonization of roots and infection sites to preclude the pathogen, parasitism of pathogen hyphae, and induction of host plant resistance are mechanisms through which these nonpathogenic strains achieved biological control (Sneh 1998).

Nonpathogenic *Fusarium* species (*F. oxysporum* and *F. solani*) provided control of *Fusarium* wilt of tomato

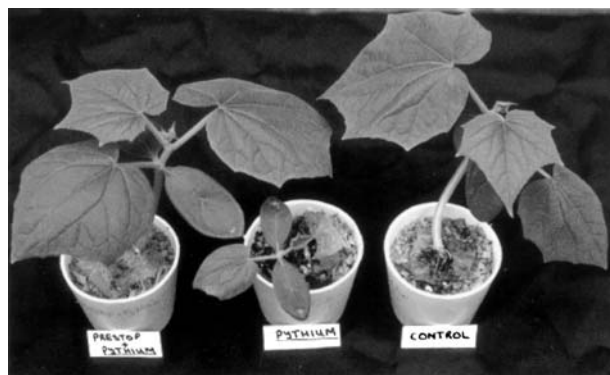


Figure 1 Effect of *G. catenulatum*, formulated as Prestop, on reducing root rot and damping-off caused by *P. aphanidermatum* on cucumber seedlings. The plant on the left received the biocontrol agent at seeding time followed by the pathogen 10 days later; the plant in the center received the pathogen only 10 days after seeding; the plant on the right is the uninoculated control. Photograph was taken at 28 days after seeding.

(Alabouvette et al. 1993; Fuchs et al. 1999; Larkin and Fravel 1998) through systemic induction of host resistance, and an increase in hydrolytic enzyme activity was reported in treated plants (Duijff et al. 1998; Fuchs et al. 1999). Induction of resistance by nonpathogenic fungi has also been reported to occur for binucleate *Rhizoctonia* species (Jabaji-Hare et al. 1999; Xue et al. 1998), *P. oligandrum* (Benhamou et al. 1997), and *Penicillium oxalicum* (DeCal et al. 1999). In plants treated with these biocontrol agents, induction of host defense responses, alterations of the plant cell wall, and enhanced expression of antifungal enzymes were reported (Benhamou et al. 1997; Jabaji-Hare et al. 1999; Xue et al. 1998). Further studies on the mechanisms by which these fungi elicit the host defense responses should provide interesting information on this group of biocontrol fungi. Formulations for biocontrol fungi such as binucleate *Rhizoctonia* species have been described (Honeycutt and Benson 2001).

3.3.1 Biotechnological Manipulations of Nonpathogenic Biocontrol Fungi

Techniques in biotechnology have been applied primarily to the nonpathogenic *F. oxysporum* strains that provide biocontrol of Fusarium wilt on a number of plant species. By creating strains expressing the marker gene β -glucuronidase (GUS) through genetic transformation, the role of competition for root colonization between pathogenic and nonpathogenic strains could be elucidated (Eparvier and Alabouvette 1994). Nonpathogenic strains differed in their ability to colonize roots and to preclude the pathogenic strains, and the use of GUS-marked strains utilizing the glyceraldehyde-3-phosphate dehydrogenase promoter provided an estimation of fungal metabolic activity on the roots (Eparvier and Alabouvette 1994).

A strain of nonpathogenic *F. oxysporum* transformed with the β -glucuronidase (*GusA*) and hygromycin B resistance (*Hph*) genes could be detected at levels as low as 1 ng of mycelia and estimates of fungal biomass on tomato roots were shown to be considerably higher compared to a plating assay method (Bao et al. 2000), indicating this was a sensitive and rapid assay for this biocontrol agent in planta. The colonization by the transformed strain of plant roots could be assessed in relation to the extent of colonization of a pathogenic strain of *F. oxysporum* (Bao and Lazarovits 2001).

3.4 Mycoparasites As Biocontrol Agents of Seedling, Root, Crown, and Vascular Wilt Diseases

Nonpathogenic fungi can act as mycoparasites, as exemplified by *P. oligandrum*, *P. nunn*, and *P. periplocum*, which are mycoparasitic on other species of *Pythium* and can reduce pathogen infection levels and reduce disease (Berry et al. 1993; McQuilken et al. 1992; Paulitz and Baker 1987). Other mycoparasitic fungi include *Stachybotrys elegans* and

Verticillium biguttatum affecting *R. solani* (van den Boogert and Velvis 1994; Tweddell et al. 1995), *Coniothyrium minitans* on *Sporidesmium sclerotiorum* (Budge and Whipps 2001), *S. sclerotivorum* on a number of sclerotial-forming soilborne fungi (Mischke 1998), and *Talaromyces flavus* on *S. rolfsii* (Madi et al. 1997). In addition, *T. flavus* was reported to produce glucose oxidase and potentially peroxide, which was lethal to sclerotia of *V. dahliae* (Stosz et al. 1996). Species of *Trichoderma* and *Gliocladium* are also known to be mycoparasitic, as discussed in previous sections of this chapter. All of these mycoparasitic fungi have been demonstrated to reduce diseases caused by a number of different pathogens on a range of vegetable crop species (Table 1).

4 BIOCONTROL OF FOLIAR-INFECTING FUNGI

Pathogenic fungi which infect the leaves and stems of developing plants may enter through senescing tissues, wounded regions, or natural openings, or may penetrate host tissues directly. These fungi can infect plants at all stages of development, and are favored by warm (20–25°C) and humid conditions. Infection results in blighting of the foliage, premature leaf senescence, and compromised plant growth and yield. Biological control agents have been described which when applied to the foliage, can reduce primary infection as well as reduce pathogen development and sporulation, and can colonize wounds and other tissues to preclude pathogen establishment or development. Some of the biological control agents can act as mycoparasites and reduce pathogen growth directly, while others may secrete hydrolytic enzymes and antifungal compounds to reduce pathogen development, or alter pathogen physiology to reduce disease-causing potential. The most widely-researched of these biocontrol agents are fungi (*Trichoderma*, *Ulocladium*, *Ampelomyces*, and *Verticillium*) and yeasts (*Aureobasidium*, *Cryptococcus*, *Rhodospodium*, and *Rhodotorula*).

4.1 Biocontrol of Gray Mold

Botrytis cinerea Pers:Fr. is an important pathogen on many vegetable crops grown under greenhouse conditions as well as under field conditions. Under high humidity conditions or when free moisture is present on the plant surface, the pathogen infects fruits, flowers, leaves, and stems causing tissue decay. This is followed by prolific sporulation of the pathogen, producing a gray mold appearance. Wounded tissues are especially susceptible to this pathogen. Much of the research activity to achieve biological control of *B. cinerea* on vegetable crops has centered around the use of *T. harzianum*, followed by *Ulocladium* spp. and a number of yeasts, as described later.

4.1.1 *Trichoderma* As a Biocontrol Agent of *Botrytis Cinerea*

Isolate T-39 of *T. harzianum* (marketed as Trichodex™) provided control of gray mold as well as a number of other fungal diseases of cucumber under commercial greenhouse conditions (Elad 2000a). *T. harzianum* T-39 was applied as part of a gray mold management program in alternation with chemical fungicides. The biocontrol agent was effective when applied in formulations containing two concentrations of the active ingredient (0.2 and 0.4 g/l), at around 10^{10} cfu/g of *T. harzianum* (Elad et al. 1993). A number of other research studies have confirmed the efficacy of *T. harzianum* strains in reducing development of *B. cinerea* on crops such as cucumber and tomato under laboratory conditions and on greenhouse-grown plants (Dik and Elad 1999; Dik et al. 1999; O'Neill et al. 1996; Utkhede et al. 2000).

Mechanisms involved in the biological suppression of infection and inoculum potential of *B. cinerea* by *Trichoderma* are numerous and variable and the involvement of two or more mechanisms has been demonstrated in several studies. Reported combinations include antibiosis with enzyme degradation of *B. cinerea* cell walls and parasitism (Bélanger et al. 1995); competition for nutrients followed by interference with pathogenicity enzymes of the pathogen or with induced resistance; and alteration of plant surface wettability combined with antibiosis (Elad 1996). Since, germinating *B. cinerea* conidia are dependent on the presence of nutrients to initiate pathogenesis, competition for nutrients is important in biocontrol. Pathogen conidial viability and germination capacity are also potentially affected by the presence of antibiotics produced by *Trichoderma* and present in the phyllosphere. Slower in action are mechanisms involving induced resistance in the host plant and production of hydrolytic enzymes that degrade *B. cinerea* cell walls. The latter has been demonstrated much more convincingly *in vitro* than in the phyllosphere. Biocontrol in established lesions and reduction of sporulation of *Botrytis* on necrotic plant tissues is a means to minimize secondary spread of pathogen inoculum. Zimand et al. (1996) also demonstrated that the presence of *T. harzianum* at the site where *B. cinerea* infects can have an adverse effect upon activity of pathogen enzymes involved in pectin degradation and host cell wall destruction, e.g., pectinase, cutinase, and pectate lyase. Since such enzymes are intimately involved in the infection process by *B. cinerea*, the effect of the biocontrol agent in reducing their activity *in vitro* and on the surface of plant leaves could also limit disease development by the pathogen (Kapat et al. 1998).

The inhibition of pathogen enzymes was proposed to be due to the secretion of serine proteases by *T. harzianum* (Elad and Kapat 1999), which could also inhibit pathogen spore germination. The presence of protease inhibitors was found to reduce the biocontrol activity. The potential role of induced plant resistance by *T. harzianum* for control of *B. cinerea* was demonstrated by De Meyer et al. (1998), wherein application of the biocontrol agent to roots or leaves of a number of different plant species was observed to provide protection

against the pathogen on leaves that were spatially separated from the site of application of *T. harzianum*. This was attributed to induction of systemic resistance that delayed or suppressed spreading lesion formation (De Meyer et al. 1998).

4.1.2 Saprophytic Fungi and Yeasts As Biocontrol Agents of *Botrytis Cinerea*

The leaf surface of plants (phylloplane) is frequently colonized by a range of saprophytic fungi and yeasts, which rely on plant nutrient exudates and a range of other carbon/nitrogen sources for their survival, e.g., damaged or senescing tissues, pollen grains, insect honeydew. If present in the same niche as plant pathogenic fungi, these saprophytes may compete with pathogens for nutrients, infection sites, or reduce growth and sporulation of the pathogen on host tissues through competition or antagonism (Fokkema 1993). Recovery of selected fungi and yeasts and reapplication to the leaf or stem surface has identified a number of potential biological control agents that can reduce diseases caused by *B. cinerea*. On onion leaf tissues, the saprophytic fungi *Alternaria alternata*, *Chaetomium globosum*, *Ulocladium atrum*, and *U. chartarum* suppressed sporulation of the pathogen significantly when applied after pathogen inoculation (Köhl et al. 1995; 1999). A monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) has been described to detect and quantify *U. atrum* in colonized plant tissues (Karpovich-Tate and Dewey 2001) and could be useful in monitoring of this biocontrol agent. Application of the saprophytic fungus *Cladosporium cladosporioides* to wounds on tomato stems was reported to reduce infection by *B. cinerea* in laboratory and greenhouse experiments (Eden et al. 1996).

The yeast-like fungi *Aureobasidium pullulans* and *Cryptococcus albidus* significantly reduced sporulation of *B. cinerea* on pruning wounds and stems of cucumber and tomato under laboratory and greenhouse conditions (Dik and Elad 1999; Dik et al. 1999). Another yeast, *Rhodospiridium diobovatum*, when applied to tomato stems, reduced lesion size due to *B. cinerea* and the treated plants yielded higher fruit when compared to the untreated controls (Utkhede et al. 2000). Both *C. albidus* and *Rhodotorula glutinis* reduced sporulation of *B. cinerea* on bean and tomato leaves and reduced disease levels (Elad et al. 1994).

4.1.3 Mechanisms of Action of Yeasts Against *Botrytis Cinerea*

Yeasts can compete effectively against *B. cinerea* for nutrients, such as glucose and fructose (Filonow 1998; Filonow et al. 1996), thereby reducing pathogen colonization of plant tissues and sporulation (Elad et al. 1994). Yeasts such as *Aureobasidium* have also been reported to produce mycotoxins in culture (Schrattenholz and Flesch 1993). Yeast cells may attach to pathogen hyphae, as demonstrated for *B. cinerea*, and cause them to degrade (Cook et al. 1997) through secretion of cell wall degrading enzymes

(Wisniewski et al. 1991). The production of cell wall degrading enzymes, such as β -1,3-glucanases has also been documented in yeasts such as *Pichia anomala* that are effective biocontrol agents against *B. cinerea* as a postharvest treatment (Jijakli and Lepoivre 1998). *In vivo* studies with *Candida saitoana* in apple demonstrated that *B. cinerea* hyphae had degenerated (El-Ghaouth et al. 1998), implicating the possible role of toxins and/or enzymes. In addition, plant cells in the vicinity of the yeasts appeared to have enhanced structural defense responses, suggesting an induction of defense in the host plant may have occurred. Stimulation of host cell defenses by the yeast *C. oleophila* was recently described (Droby et al. 2002).

4.1.4 Biotechnological Techniques Applied to Yeasts

Yeasts with biocontrol potential against gray mold have been characterized using molecular techniques to provide a method to distinguish between closely-related strains and to identify and monitor survival of strains after application (Schena et al. 2000; 2002). These techniques include arbitrarily primed polymerase chain reaction (AP-PCR), random amplified polymorphic DNA (RAPD-PCR) analysis, and sequence-characterized amplification region (SCAR) analysis. In addition, transformation of the yeast *Metschnikowia* with green fluorescent protein (GFP) was achieved and colonies could be visualized under epifluorescence (Nigro et al. 1999). The transformed strains behaved similarly to the wild-type strains in biocontrol activity against *B. cinerea* and in growth rates. Another yeast, *A. pullulans*, was also transformed with GFP and colonies were readily visible on apple leaf surfaces when subjected to fluorescence and could be quantified (Wymelenberg et al. 1997).

Genetic transformation of the yeast *Saccharomyces* to express a cecropin A-based peptide with antifungal activity was recently described (Jones and Prusky 2002). The transformants inhibited the growth of *Colletotrichum* and reduced fungal decay of tomato fruits when applied prior to pathogen inoculation. The expression of the antifungal peptide in the biological control agent suggests a new approach for disease control.

4.2 Biocontrol of Leaf and Stem Blights

Didymella bryoniae (Auersw.) Rehm (anamorph *Phoma cucurbitacearum* Fr.:Fr.) Sacc. is an important pathogen on greenhouse- and field-grown cucumbers and other cucurbits, and causes the disease gummy stem blight. The disease is favored by warm, humid conditions and the pathogen infects stems, fruit, leaves, and flowers of susceptible plants, especially through wounded or senescing tissues, and natural openings such as stomata and hydathodes. There are few reports on the potential of using biological control agents to control this disease. Utkhede and Coch (unpublished) applied the yeast *R. diobovatum* and the biocontrol agent

G. catenulatum J1446 as preventative treatments to wounded stem tissues of cucumber followed by inoculation with the pathogen. They demonstrated that both microbial agents significantly reduced disease development when compared to plants treated with water. Anthracnose disease of cucumber, caused by *Colletotrichum magna*, was reduced when a nonpathogenic mutant was applied to seedlings to achieve colonization and induction of defense responses that subsequently protected treated plants against the pathogen (Redman et al. 1999).

4.3 Biocontrol of Powdery Mildews

Powdery mildew fungi are obligate parasites of plants that derive nutrients and water from their host, thereby reducing growth and yield through the acquisition of photosynthates. The fungi penetrate into the epidermis directly and establish a parasitic relationship with the plant host through the formation of haustoria, the nutrient-absorbing structures. Mycelial growth and sporulation occur on the surface of leaves and stems, resulting in a white fuzzy mildew appearance.

Over the years, powdery mildew diseases have been managed through the use of chemical fungicides and genetic resistance, but recent reports have highlighted the potential of biological control methods. Fungal and yeast biological control agents have been described which can reduce sporulation and growth of mildew pathogens, thereby minimizing their damaging effects to host plants. The fungal biocontrol agents are mostly mycoparasites, while the yeasts produce antibiotics and hydrolytic enzymes that cause the mildew hyphae and conidia to collapse and be rendered nonviable.

4.3.1 Fungi As Biological Control Agents of Powdery Mildews

Verticillium lecanii has been described as a mycoparasite of powdery mildew fungi as well as a pathogen of insects and it has been developed as a biocontrol agent of insects on greenhouse crops. Strains of *V. lecanii* differed in their level of antagonism against the powdery mildew pathogen of cucumber, *Sphaerotheca fuliginea*, under laboratory conditions (Askary et al. 1998). Application to cucumber leaves prior to mildew infection and incubation under high (>95%) relative humidity conditions reduced mildew development (Verhaar et al. 1997). The high humidity requirement for growth of this mycoparasite was reduced by the addition of an oil formulation (Verhaar et al. 1999). Infection of *S. fuliginea* by *V. lecanii* resulted in disorganized cytoplasm and plasmalemma disruption, possibly due to chitinase enzyme activity (Askary et al. 1997).

Another mycoparasite, *Ampelomyces quisqualis*, has been extensively studied as a biocontrol agent of powdery mildew of cucumber. The mycoparasite infects the mildew pathogen and forms pycnidia in association with colonized mycelium,

reducing growth and sporulation of the pathogen. Cells of the mycoparasite grow inside the mildew hyphae, gradually causing them to degenerate. High levels of β -1,3-glucanase activity were reported in *A. quisqualis* (Rotem et al. 1999) and exposure of mildew hyphae to the enzymes caused them to degrade.

A commercially available formulation of *A. quisqualis* AQ10 has been extensively evaluated against powdery mildew development. On cucumbers grown in the greenhouse, AQ10™ was very effective in reducing mildew development (Elad et al. 1998). On field-grown cucurbits, AQ10™ also suppressed mildew development and increased yield when compared to the nontreated plants (McGrath and Shishkoff 1999).

Molecular techniques have been used to characterize strains of *A. quisqualis*. The RFLP analysis of the nuclear rDNA ITS region and sequence analysis among a worldwide collection of isolates revealed considerable intraspecific variation (Kiss 1997; Kiss and Nakasone 1998). Isolates of the same genetic background were found in widely different areas and genetically different isolates could be found in a given area.

4.3.2 Yeasts As Biological Control Agents of Powdery Mildew

Pseudozyma (Sporothrix) flocculosa is a yeast-like fungus with demonstrated biocontrol activity against powdery

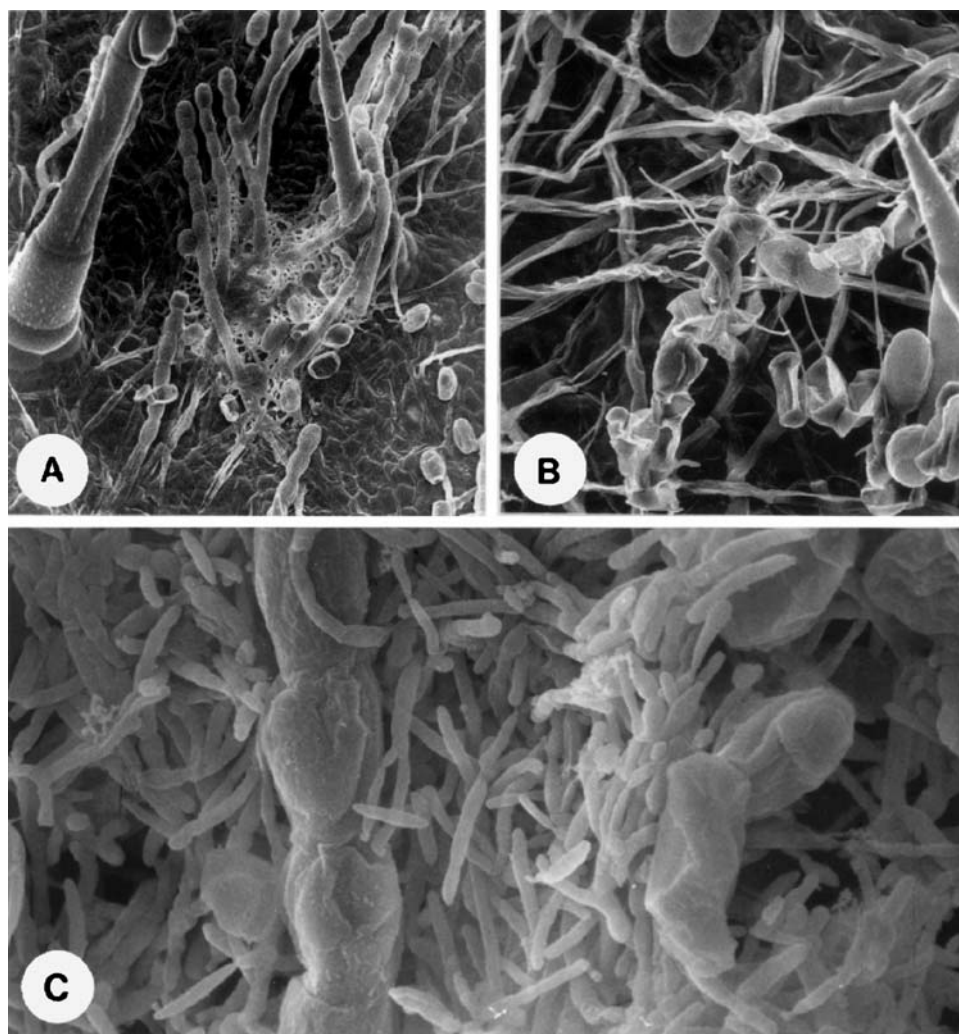


Figure 2 Effect of *Tilletiopsis pallescens* on development of powdery mildew (*S. fuliginea*) on cucumber leaves. (A) A mildew-infected leaf showing chains of conidia and mycelium. (B) A mildew colony treated with a 3-day-old liquid culture of *T. pallescens*. Note collapsed conidia and mycelium. Photograph was taken 2 days following treatment. (C) Spore masses of *Tilletiopsis* adjacent to mildew conidia. Note intact mildew conidia on left and collapsed conidia on the right.

mildew fungi, especially on cucumber and rose (Bélanger and Benyagoub 1997; Belanger et al. 1994). Cytochemical investigations have shown that the yeast induces a rapid collapse of mildew spores and hyphal cells (Hajlaoui et al. 1992). Extracellular fatty acids with antifungal properties were produced by *P. flocculosa* and reported to be the principle mode of action in biological activity against powdery mildew (Benyagoub et al. 1996), by disrupting the cytoplasmic membrane in a range of fungi (Avis and Bélanger 2001). A fungicide-tolerant strain of the yeast was selected which could be used in conjunction with chemical control methods to reduce powdery mildew development (Benyagoub and Bélanger 1995). In a comparative study of three biological control agents against powdery mildew of cucumber, i.e., *V. lecanii*, *A. quisqualis*, and *P. flocculosa*, it was shown that *P. flocculosa* gave the best disease control (Dik et al. 1998).

Molecular techniques have been used to characterize strains of *Pseudozyma flocculosa* (Avis et al. 2001b). Ribosomal DNA sequences and random amplified microsatellites were used to distinguish among different strains of this species and to develop isolate-specific markers to monitor spread and confirm genetic fidelity of the strains. A strain of *P. flocculosa* has been formulated and produced commercially under the name Sporodex™ for use in control of powdery mildew on a number of crops grown under greenhouse conditions.

Species of *Tilletiopsis* are saprophytic yeast-like fungi that occur as epiphytes on the leaf surface of various plant species and which have been demonstrated to have biological control activity against powdery mildew diseases (Hijwegen 1992; Knudsen and Skou 1993; Urquhart et al. 1994). Scanning

electron microscopic studies have revealed that mildew hyphae and spores appeared collapsed after treatment with *Tilletiopsis* (Figure 2) (Urquhart and Punja 1997). It was postulated that extracellular antifungal compounds were involved in biocontrol activity that included fatty acid esters and hydrolytic enzymes (Urquhart and Punja 2002). Various species of *Tilletiopsis* have demonstrated biological control activity, including *T. albescens*, *T. minor*, *T. pallescens*, and *T. washingtonensis* (Table 2). These species could be distinguished using RAPD analysis of PCR-generated DNA with random primers (Urquhart et al. 1997). Intraspecific variation was also noted and DNA fingerprints were generated for some isolates that could be useful for monitoring the distribution and spread of certain isolates.

5 CONCLUSIONS

The numerous reports of success in the utility of fungal and yeast biological control agents to reduce fungal diseases on vegetable crops illustrate the potential of this approach for disease management. In addition, the applications of techniques in biotechnology are providing numerous examples of how these biocontrol agents can be characterized, monitored, and investigated in more depth. However, there are unique requirements in working with microbial biocontrol agents that must be recognized if this approach to disease control is to be successful.

Environmental conditions, particularly temperature and moisture, can greatly influence the degree to which fungal and yeast biological control agents can affect fungal diseases on vegetable crops, even in greenhouse environments. Therefore,

Table 2 Examples of fungal and yeast biological control agents that reduce foliar diseases on vegetable crops caused by pathogenic fungi

Biocontrol agent	Target pathogen and host	References
<i>T. harzianum</i>	<i>B. cinerea</i> on cucumber	Dik and Elad (1999) and Elad et al. (1993; 1998)
<i>T. harzianum</i>	<i>B. cinerea</i> on tomato	Dik and Elad (1999), Migheli et al. (1994), and Utkhede et al. (2000)
<i>T. harzianum</i>	<i>C. fulvum</i> on tomato	Elad et al. (2000a)
<i>T. harzianum</i>	<i>S. fuliginea</i> and <i>S. fusca</i> on cucumber	Elad et al. (1998; 2000b)
<i>G. catenulatum</i>	<i>D. bryoniae</i> on cucumber	Utkhede and Coch (unpublished)
<i>A. quisqualis</i> AQ10	<i>S. fusca</i> on cucumber	Elad et al. (1998)
<i>A. pullulans</i>	<i>B. cinerea</i> on tomato and cucumber	Dik and Elad (1999)
<i>C. albidus</i>	<i>B. cinerea</i> on bean, tomato, and cucumber	Dik and Elad (1999) and Elad et al. (1994)
<i>R. glutinis</i>	<i>B. cinerea</i> on bean and tomato	Elad et al. (1994)
<i>R. diobovatum</i>	<i>B. cinerea</i> on tomato	Utkhede et al. (2000)
<i>C. cladosporioides</i>	<i>B. cinerea</i> on tomato	Eden et al. (1996)
<i>S. flocculosa</i>	<i>S. fuliginea</i> on cucumber	Dik et al. (1998)
<i>T. albescens</i> , <i>T. minor</i> , <i>T. pallescens</i> , and <i>T. washingtonensis</i>	<i>S. fuliginea</i> on cucumber	Hijwegen (1992), Knudsen and Skou (1993), Urquhart and Punja (1997), and Urquhart et al. (1994)

careful monitoring and recording of environmental variables is a requisite. The biological control agents are generally most effective when applied as a preventative treatment, prior to or at the onset of disease, and multiple applications may be needed to provide longer-term disease suppression. At high levels of disease pressure, biological control agents can be anticipated to perform less well. Some of the agents may be used in combination with, or in alternation with, chemical fungicides if it can be demonstrated that their survival is not adversely affected. Similarly, it may be possible that combinations of biocontrol agents may be more effective than single organisms although little research has been done in this area. Biological control agents that affect more than one disease should have greater market potential than those that specifically target a particular disease. It is not clear whether different plant hosts may have an influence on the efficacy of these biocontrol agents.

Notwithstanding these conditions, the use of fungal and yeast biological control agents has generated significant interest in both the scientific research and product development arenas to ensure that commercially viable products will continue to be brought to market.

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Control of Postharvest Diseases of Fruits Using Microbes

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1 INTRODUCTION

Losses from postharvest diseases of fruits have been substantial at the storage, wholesale, retail, and consumers levels. The total losses are very difficult to establish because research has generally considered only one or two levels, and little work has been done to determine losses at the consumer level. Nevertheless, in the United States, losses are estimated to range from 5% for citrus to as much as 20% for strawberries (Cappellini and Ceponis 1984; Eckert and Ogawa 1985). Most of the fruit decay results from infection through wounds made during harvest and postharvest handling, but for some fruits, infection takes place in the orchard during the growing season, and remains latent. As fruit mature in storage the pathogens become active again and invade fruit tissue. A variety of approaches have been used to reduce postharvest fruit decays, including sanitation to reduce pathogen inoculum, gentler handling of fruit to reduce wounding (Sommer 1982), physical treatments such as hot water dips and hot air treatments that kill pathogens (Falik et al. 1995; Lurie et al. 1998), storing produce at low temperatures or in modified atmosphere which stop or reduce growth of the pathogens (Sommer 1982), treating fruit with chemicals that enhance natural resistance (El-Ghaouth 1998), with synthetic fungicides (Eckert and Ogawa 1985; Eckert and Ogawa 1988), and, more recently, with biocontrol agents (Droby et al. 1998; Janisiewicz and Jeffers 1997; Janisiewicz and Korsten 2002; Korsten et al. 1995; Usall et al. 2001). Fungicides have been, by far, the most widely used remedy against fruit decay because they are easy to apply and generally, one fungicide is effective against most of the pathogens on a specific crop. Storage of some fruits for extended periods, e.g., citrus fruits, is totally dependent on the use of fungicides. But postharvest use of synthetic fungicides

has been increasingly curtailed by the perceived hazard to humans and the environment. This has resulted in new regulations restricting or eliminating their use in this country and abroad. It has become increasing difficult to find and register new fungicides to replace those to which postharvest pathogens have developed resistance (Gullino and Kuijpers 1994; Ragsdale and Sisler 1994). Thus, there has been a need to find effective alternatives to synthetic fungicides. None of the alternative methods developed during the past two decades have had the broad spectrum of activity as synthetic fungicides. Recently, biological control has emerged as an alternative (Janisiewicz and Korsten 2002). The full potential of biocontrol has not yet been realized because the mechanisms of biocontrol have not been explained. A fuller understanding of the antagonistic mechanisms will eventually help manipulate and improve the biocontrol system. Although this method has some limitations, these limitations can be addressed by combining biological control with other alternative methods (Conway et al. 1999; El-Ghaouth et al. 2000b; Janisiewicz et al. 1998; Smilanick et al. 1999). In this chapter, the key elements in the development of biological control of postharvest diseases (BCPD) of fruits, and the current status and future prospects of BCPD of fruits using examples of fungal and bacterial antagonists are discussed.

2 PATHOGENS TARGETED FOR BCPD OF FRUITS

The BCPD of fruits can be approached from the perspective of the host plant (different fruits), habitat for the microorganisms (wound, intact surface), and the pathogen's strategy used to infect fruit. The pathogen's strategy has been emphasized most frequently because many economically

important postharvest diseases of fruits are caused by necrotrophic pathogens (Dennis 1983). These pathogens invade mainly through wounds, and require nutrients for spore germination and initiation of the pathogenic process, which makes them vulnerable to competition for nutrients from surrounding microorganisms. Other mechanisms of biocontrol described later in this chapter may also be involved, but the prevailing evidence suggests that their role is secondary (Janisiewicz and Korsten 2002). Incipient or latent infections that generally occur in the field are less prone to biological control because the pathogen has already established a parasitic relation with the host. However, these pathogens can be controlled by antagonists that prevent infection in the field, perpetuate latency by removal of nutrients from areas surrounding the appressoria, or perhaps those that can inhibit pathogen development by the production of antifungal substances or by direct parasitism (Koomen and Jeffries 1993; Korsten and Jeffries 2000; Leibinger et al. 1997).

The greatest progress in BCPD of fruits has been made against typical wound-invading necrotrophic postharvest pathogens such as *Penicillium expansum* which causes blue mold of apple, pear, and cherries, *Botrytis cinerea* which causes gray mold of pome fruits (Janisiewicz and Jeffers 1997), *P. italicum* and *P. digitatum* which causes blue and green-mold of citrus fruits, respectively (Droby et al. 1998), and against the wound invading phase of brown rot decay of stone fruits caused by *Monilinia fruticola* (Pusey et al. 1988). Although the likelihood of infection is dependent on the concentration of fungal spores, the biocontrol strategy has always focused on the preemptive colonization of the wounds by the antagonist to prevent infection, and not on reduction of the pathogen inoculum. Thus, the control of these decays was achieved by the application of antagonists to wounds, simultaneously with the pathogen or shortly after the infection took place. Other potential candidates for this type of biological control are pathogens invading through cut stem of bananas, mangos, and papayas (Eckert 1991). Since the development of the pathogen depends on fruit maturity and the environment, these factors have been critical in the pathogen-antagonist interaction and resulting biological control.

Significant successes were achieved with biocontrol of latent infections caused by *Colletotrichum* spp. on mango and avocado (Korsten and Jeffries 2000), and to a lesser extent by *B. cinerea* on strawberries (Helbig 2002; Ippolito et al. 1998; Peng and Sutton 1990; Takeda and Janisiewicz, unpublished results). Biological control of these diseases must start in the field, relies on multiple application of the antagonist, and is generally more difficult to achieve.

3 ANTAGONIST SELECTION

The first successful attempts in BCPD of fruits, which stimulated research in postharvest biological control, used soil isolated bacterium, *Bacillus subtilis*, to control brown rot

of stone fruits caused by *M. fructigena* (Pusey and Wilson 1984). Subsequent works focused on screening natural microflora from the aerial surfaces of apple and pear trees for antagonistic activity against decays caused by *P. expansum* and *B. cinerea* (Janisiewicz 1987). This resulted in the isolation of many bacteria and yeasts that were effective in controlling fruit decays caused by these pathogens. Isolation from the fruit surfaces has become a standard practice and is the most efficient source of antagonists against postharvest fruit pathogens of temperate, subtropical, and tropical fruits (Adikaram and Karunaratne 1998; Arras 1993; Chalutz et al. 1988; Chand-Goyal and Spotts 1996; Droby et al. 1999; Guinebreiere et al. 2000; Huang et al. 1992; Kanapathipillai and bte Jantan 1985; Lima et al. 1998; Qing and Shiping 2000; Teixidó et al. 1998a; Testoni et al. 1993; Zahavi et al. 2000). A variety of enrichment procedures, employing either fruit juice or tissue, have been used to isolate microorganisms best suited to colonize wounded fruit tissue (Janisiewicz 1991; 1996; Wilson et al. 1993). The enrichment procedures appear to favor isolation of the resident fruit microflora, with the yeasts being isolated most frequently, followed by bacteria. Filamentous fungi have been isolated only sporadically (Janisiewicz 1996; Wilson et al. 1993). The rapid colonization of wounds by yeasts is necessary for preemptive exclusion of the wound-invading pathogens. The number of species that are residents on a specific kind of fruit is limited, and reports from various laboratories worldwide increasingly describe biocontrol potential of the same antagonist species isolated at different locations (Chand-Goyal and Spotts 1996; 1997; Falconi and Mendgen 1994; Ippolito et al. 2000; Janisiewicz et al. 1994; 2001; Leibinger et al. 1997; Lima et al. 1998; McLaughlin et al. 1992; Roberts 1990; Wisniewski et al. 1988). Recent studies; however, indicate great diversity within an antagonist species, even at a single geographical location, with regard to effectiveness in controlling fruit decays and other factors important in commercializing a biocontrol agent (Janisiewicz et al. 2001; Schena et al. 1999). Thus, investigating the same species of the antagonist at various locations may lead to finding an antagonist with superior attributes. An effective antagonist may also be found by screening starter cultures used for food products (Pusey 1991), various culture collections (Filonow et al. 1996), and even by exploring an aquatic environment, as is the case with bacteriophages used against soft rotting bacteria (Eayre et al. 1995).

In addition to being effective in controlling fruit decays, antagonists should have certain attributes to make them good candidates for commercialization. These include: compatibility with postharvest practices (storage temperatures, relative humidity-RH, storage atmosphere with elevated CO₂ and reduced O₂, handling in water, heat drying tunnels, etc.), treatments and additives (waxes, antioxidants, flotation salts), ability to grow efficiently in a commercially used media for mass production, ease of formulation, and the lack of potentially deleterious effects on human health that would disqualify them from being approved by regulatory agencies. Human safety, in particular, necessitates a thorough

approach in identifying an antagonist. Misidentification may result in abandonment of commercial development of the antagonist, and, if not detected early, may be very costly.

4 MECHANISMS OF BIOCONTROL

Various mechanisms of biocontrol have been suggested for antagonists effective in BCPD of fruits and often more than one mechanism was implicated for a single antagonist. In no case, however, was the biocontrol mechanism fully explained. The putative mechanisms included competition for limiting nutrients and space, lysis, induced resistance, direct parasitism, and production of inhibitory substances. Attachment of antagonists to a fungal hyphae was observed in some antagonist-pathogen interactions, but its role remains largely speculative (Arras et al. 1998; Wisniewski et al. 1989; 1991). The main reasons for the limited knowledge in mechanism of biocontrol have been a lack of appropriate methods to study microbial interactions in wounds of fruit, and the fact that progress in BCPD was driven by advances in microbial ecology of the antagonists. However, recent advances in microbial sensing of nutrients on plants (Lindow et al. 2002), molecular approaches (Bassett and Janisiewicz 2001; Jijakli et al. 2001; Jones and Prusky 2002; Yehuda et al. 2001), and a method allowing separation of competition for nutrient and space using natural substrates (Janisiewicz et al. 2000) may lead to better explanation of the significance of various biocontrol mechanisms. Progress in microbial ecology of the antagonists led the commercialization of BCPD of fruits in a relatively short period of time, but further expansion will greatly depend on achieving the full potential of BCPD, for which knowledge of the mechanisms of biocontrol will be essential.

Bacterial antagonists such as *Bacillus* spp. (Gueldner et al. 1988) or *Pseudomonas* spp. (Bull et al. 1998; Janisiewicz and Roitman 1988) produce a variety of antifungal compounds in artificial media, which by themselves can provide effective control of postharvest decays of fruits (Bull et al. 1998; Janisiewicz et al. 1991; Takeda and Janisiewicz 1991). But the role of these antifungal compounds as the biological control mechanism is uncertain, because they either can not be detected in fruit wounds after inoculation with the antagonist (Bull, personal communication), or pathogen mutants resistant to these inhibitory substances are still controlled by the antagonists (Smilanick, personal communication).

Yeast antagonist such as *Pichia anomala* strain K (Gravesse et al. 1998; Jijakli and Lepoivre 1998), *P. guilliermondii* (Arras et al. 1998; Wisniewski et al. 1991), or yeast like *Aureobasidium pullulans* (Castoria et al. 2001) effective in controlling gray mold of apples, produce β -1,3-glucanase, which caused lysis of the *B. cinerea* hyphae. Production of this enzyme by *P. anomala* strain K was stimulated in the presence of cell wall preparations of *B. cinerea* in apple wounds resulting in improved biocontrol (Gravesse et al. 1998; Jijakli and Lepoivre 1998). This

enzyme also increased in apple wounds treated with the *A. pullulans* cells, but how much of this increase could be attributed to production by the antagonist, on the fruit itself, was not resolved (Castoria et al. 2001). This antagonist can also produce aurebasidins, antibiotics whose role has not been determined. The antagonistic yeast, *Candida saitoana*, effective in reducing decays of citrus and apple, induced chitinase activity in apple (Wilson and El Ghaouth 1993). *C. oleophila*, used in the commercial product Aspire, induced resistance responses such as production of chitinase, β -1,3-endoglucanases, PAL, phytoalexins scoparone and scopoletin and ethylene in flavedo tissue of grapefruit (Droby et al. 2002). The contribution of these induced resistance responses to biocontrol was not determined. The yeast *C. famata*, effective in reducing green mold caused by *P. digitatum* on oranges, increased the phytoalexins scopoletin and scoparone 12-fold in fruit wounds after four days, but the role scoparone in biocontrol is uncertain due to its relatively slow production (Arras 1996). The antagonists *Cryptococcus laurentii* and *Sporobolomyces roseus*, effective against gray mold of apple, utilized the apple volatile, butyl acetate, which stimulated germination and adhesion to membranes of *B. cinera* conidia (Filonow 1999; 2001). The significance of these phenomena in the biological control was not established due to the technical difficulties in conducting this type of experiment in fruit wounds. When these antagonists were applied to harvested fruits, they colonized fruit wounds rapidly, and competition for limiting nutrients and space was suggested as an important biocontrol mechanism. Removal of limiting nutrients may also be responsible for maintaining the dormancy of *Colletotrichum* spp. appresoria on fruit treated with antagonistic *Bacillus* spp. (Korsten and Jeffries 2000).

5 IMPROVING BCPD

The goal of the biocontrol improvement program is to realize the full potential of biological control. This may be accomplished by more extensive strain selection of the same antagonist species, as indicated earlier in the case of *M. pulcherrima*, by manipulating antagonists and/or their environment, and by applying the antagonists before harvest in addition to one after harvest.

Postharvest application of the antagonists mixture of *P. syringae* and *S. roseus* (Janisiewicz and Bors 1995), and *C. sake* and *Pantoea agglomerans* (Nunes et al. 2002) improved efficacy of biocontrol of blue mold of apples compared to the individual antagonist. An orchard application of a mixture of *A. pullulans* with *Rhodotorula glutinis* was more effective than the individual antagonists, and suppressed apple decays caused by *Penicillium* spp., *B. cinerea*, and *Pezizula malicorticis* after harvest to the same level as the commonly used fungicide Euparen (Leibinger et al. 1997). Nutrient utilization profile of individual antagonists was successfully used to develop antagonist mixtures with a minimum of nutrient overlap between the antagonists and

resulted in biocontrol of blue mold of apples that was superior to the individual antagonists (Janisiewicz 1996).

Physiological manipulation has been focused on improving antagonist fitness by growing them under various conditions that improved resistance to desiccation and survival on the fruit. This is of particular importance to antagonists that are applied in the orchard for control of postharvest decays. *C. sake* cells grown under water stress caused by addition of glucose or glycerol increased after application to apple trees, while those grown on unmodified media did not (Teixidó et al. 1998b). This yeast was more water-stress tolerant when grown on a molasses-based medium than on a medium where water activity (a_w) was modified by the addition of NaCl (Abadias et al. 2001b). In preparing a freeze-dried formulation, viability of the *C. sake* cells was best maintained when 10% skim milk was combined with other protectants such as lactose, glucose, fructose, or sucrose (Abadias et al. 2001a). In general, the highest viability of the *C. sake* cells occurred when the protection and rehydration media were the same.

Control of blue mold of apples was improved by the addition of the amino acids L-asparagine or L-proline to the *P. syringae* antagonist treatment suspensions (Janisiewicz et al. 1992). These amino acids were selected after screening various C and N sources for their effect on the antagonist and pathogen growth. Both amino acids increased population of the antagonist more than 10-fold in the fruit wounds. The addition of the glucose analog, 2-deoxy-D-glucose, to the antagonistic yeasts *S. roseus* and *C. saitoana* significantly improved decay control on apple and citrus, respectively (El-Ghaouth et al. 2000c; Janisiewicz 1994). 2-deoxy-D-glucose can be up taken by the pathogens but it cannot be metabolized as energy source, resulting in reduced pathogen growth, which gives the advantage to the antagonists, and improves biocontrol. Ammonium molybdate stimulated population of the antagonistic yeast *C. sake* (CPA-1) and improved control of blue mold of apple and pear after harvest (Nunes et al. 2001a). This nutrient also has fungicidal activity and inhibited germination of *P. expansum* and *B. cinerea* spores *in vitro*, and reduced blue mold, gray mold, and *Rhizopus stolonifer* decay of apple in pre- and postharvest applications (Nunes et al. 2001b).

Use of genetic manipulation to improve BCPD has great potential, but little research has been done in this area. The appearance of decay symptoms on avocado fruit was delayed when the fruit were dipped in a suspension containing a reduced-pathogenicity mutant of the avocado pathogen *Colletotrichum gleosporioides* (Yakoby et al. 2001). This mutant was generated by restriction enzyme-mediated integration (REMI) transformation, and induced natural resistance of avocado by increasing production of the antifungal diene from 700 to 1,200 $\mu\text{g/g}$ fresh weight 9 days after inoculation. *Saccharomyces cerevisiae* transformed with a cercopin A-based peptide, that inhibits germination of *C. cocodes* at 50 μM , was able to inhibit the growth of germinated spores, and inhibited decay development caused by *C. cocodes* on wounded tomato (Jones and Prusky 2002).

This work demonstrated that microorganisms that colonize fruits can be used as vehicles for providing decay control which may include various biocontrol traits.

6 INTEGRATING BCPD WITH OTHER ALTERNATIVES

Although BCPD can provide levels of control that are commercially acceptable, the performance margin of biocontrol is generally lower than for fungicides. For example, higher concentrations of the antagonist must be used to achieve the same control of decay as fruit mature. To increase the performance margin of biocontrol, attempts have been made to integrate biocontrol with other alternatives to synthetic fungicide methods that were developed mainly during the past two decades (Conway and Sams 1983; Falik et al. 1995; Smilanick et al. 1995; 1997; 1999; Smoot and Melvin 1965; Spotts 1984; Spotts and Chen 1987; Tukey 1993). These methods alone did not provide commercially acceptable control of fruit decay, but in combination with biocontrol increased its performance margin.

Infiltration of apples with calcium chloride alone reduced blue mold decay by approximately half (Conway and Sams 1983), but in combination with the antagonist, *P. syringae*, resulted in greater reduction of fruit decay than either treatment alone (Janisiewicz et al. 1998). The effects of calcium treatment were greatest on more mature fruit, inoculated after 3 or 6 months in storage, when the effectiveness of biocontrol declines (Conway et al. 1999). Combining biocontrol with a calcium treatment complements each other to overcome the shortcomings of each, and may allow for reduced amounts of both products to be used without compromising decay control. In addition, applying lower calcium concentrations would reduce potential calcium injury, while maintaining other benefits, including alleviating storage maladies, such as bitter pit. The addition of calcium chloride to the yeast antagonist *Candida* sp. also improved control of blue mold and gray mold on apples (McLaughlin et al. 1990; Wisniewski et al. 1995).

Treating apples with hot air (4 d at 38°C) may virtually eliminate blue mold of apple but it has no residual effect, and any inoculation with pathogens following heat treatment results in decay (Falik et al. 1995; Lurie et al. 1998). Combining antagonistic yeasts or bacteria with heat treatment improved control of blue mold on apples (Conway et al. 1999). The heat treatment eradicated *P. expansum* infections up to 12 h after inoculation, and yeast and bacterial antagonists provided the residual effect. The heat treatment complemented the lack of eradication activity of the antagonists, the major shortcoming of BCPD. It may have additional benefits of eradicating pathogens from fruit bins and storage rooms (Douglas 1998).

Substances generally regarded as safe (GRAS), such as sodium carbonate, sodium bicarbonate, ethanol, acetic acid, hydrogen peroxide, chitosan, or some edible coatings can

reduce pathogen germination and growth. They are acceptable to the consumers, and in contrast to synthetic fungicides, do not have the prospect of a lengthy and costly approval process from regulatory agencies. For example, sodium carbonate has been used to treat lemons in commercial packinghouses (Smilanick et al. 1999). Combining 3% sodium carbonate with the antagonist *P. syringae* ESC-10 was superior to the individual treatments in controlling green mold on citrus (Smilanick et al. 1999). This compound also improved control of blue mold and gray mold of oranges in combination with antagonist *P. agglomerans* (CPA-2) (Teixido et al. 2001). Sodium carbonate has up to 24 h of eradication activity, but little residual activity, which, like heat treatment, complements biocontrol. Treatment of lemons with 10% ethanol reduced green mold to less than 5% (Smilanick et al. 1995). The addition of 10% ethanol to suspensions of *S. cerevisiae* strains 1440 and 1749, which had little biocontrol activity, reduced gray mold decay of apple from more than 90% to close to 0% (Mari and Carati 1998). Chitosan and its derivatives can reduce fungal growth and induce resistance responses in harvested fruits and vegetables (Allan and Hadwiger 1979; El-Ghaouth 1998). The addition of 0.2% glycolchitosan to a suspension of the antagonist *C. saitoana* increased control of green mold of oranges and lemons, and gray and blue mold of apples over that of the antagonist alone (El-Ghaouth et al. 2000a, b).

There are many more possibilities for combining biocontrol agents with GRAS substances or other nonfungicidal treatments. The above examples of improving biocontrol and integrating biocontrol with other nonfungicidal treatments demonstrate that biocontrol is amenable to manipulation and can be easily integrated with various decay control measures resulting in additive or synergistic effects. Strategies must be developed for the integration of various treatments in order to maximize decay control (Conway et al. 1999). Various control measures may be applied in succession and these applications may be customized to fit different postharvest practices.

7 COMMERCIALIZATION OF BCPD

Finding an industry partner is essential for the commercialization of any antagonist for postharvest biocontrol. Formulation, pilot tests, toxicology tests, and registration of the product are expensive and entail more than most research programs in government laboratories or academia are equipped to handle. Although there are examples of commercialization of biocontrol products for plant diseases control by individual scientists, especially from academia, they generally involve creation of a private company that can generate venture capital. USDA/ARS has developed a number of useful vehicles that allow private industry to commercialize inventions created in government laboratories by working jointly with the government scientists. These include simple Material Transfer Agreements, or

Memorandums of Understanding, which allow private industry the initial exploration of the commercial potential of an invention, or more definitive, Cooperative Research and Development Agreements (CRADA) that specify the role of each party in the commercial development and ownership of the final product. Having a private industry partner warrants closer scrutiny of the economic impact of the disease, importance of the disease to be controlled, and the potential for biocontrol to be competitive with the other control measures in terms of efficacy and cost. A CRADA was essential in the commercialization of BioSave™ by EcoScience Corp., which is based on *P. syringae*, and Aspire™ by Ecogen-Israel Partnership, Ltd., which is based on *C. oleophila*. For example, under the CRADA, mass production by fermentation and biomass yield were determined for the *P. syringae* antagonist first, and EcoScience Corp. investigated the potential for registration and formulation of the antagonist. This was followed by joint biocontrol feasibility and up-scale tests under simulated commercial conditions, biocontrol tests with various formulations developed by EcoScience Corp., and the pilot test with the final formulation. EcoScience Corp. developed safety data and registered the product. Production, marketing, and quality control were conducted by EcoScience Corp. Also, essential for the success of BioSave was the well-developed distribution of the product, skillful technical assistance, and rigorous quality control. This approach could be used as a model for successful public/private sector cooperation in the commercialization of other antagonists for BCPD (Stack 1998).

The commercial development of Avogreen in South Africa, which contains *B. subtilis* and is applied in the field for the control of postharvest diseases of avocado, followed a slightly different path (L. Korsten, personal communication). Once registered, the approach was “to make the system work in the hands of the farmer.” First, growers tested the product on a limited scale and were encouraged to integrate the product with existing copper sprays. By slowly phasing in biocontrol, growers gained confidence in the product. They were provided with technical support to calculate dosages, and develop suitable spray schedules adjusted for their spray equipment, cultivars used, age, and history of their orchards and disease profiles. Different formulations were developed to adapt biocontrol to different mixing systems, and for integration with existing chemicals and application methods. Application guidelines were subsequently developed for all possible application systems and for different customer needs. The wettable carrier was found to be more desirable from a production point of view because it sustained excellent cell and biomass densities, had acceptable shelf life, and was more economical. Rigorous quality control has been an integral part throughout the development of this product.

Marketing biological control products requires extensive knowledge in the fields of biological- and integrated control, production systems, and microbial ecosystems. The effectiveness of implementing biocontrol alone or in integrated systems will largely depend on knowledge of the product,

thorough understanding of its complexity, and transferring this knowledge to the market place. These aspects are often neglected in the commercialization of biocontrol.

8 CONCLUSIONS

Work conducted on BCPD demonstrates that in some cases biological control alone can provide adequate decay control, but in others it must be integrated with additional control measures. BCPD is compatible with many alternatives to fungicide treatment, can be easily adapted to current postharvest practices, and be used in a cascade system, where each additional control measure further reduces fruit decay. Recent advances in physiological and genetic manipulation of the yeast biocontrol agents and the development of superior antagonist mixtures that led to significant increases in the efficacy of BCPD are only the tip of the iceberg in showing what can be accomplished with the postharvest biocontrol system. They are also indicative that, in the future, there will be additional situations where biocontrol treatment alone will be adequate for the control of postharvest fruit decays. Greater effort is needed to identify circumstances where currently available biocontrol can be used alone. This includes not only replacing a fungicide treatment but also instances where no fungicides are registered for postharvest use and losses due to decay are significant, e.g., *Botrytis* rot on pomegranates designated for processing. There are an increasing number of situations where a fungicide is registered for postharvest use in the United States but not in other countries, particularly in Europe. This may restrict export markets or increase losses if major decay control measures are not implemented for fruit designated for export. In many of these instances, biological control can be an acceptable treatment. Currently, the European Community is sponsoring a large international project on biological control of postharvest diseases of fruits as an alternative to synthetic fungicide treatment after harvest. Experience with the commercial products for BCPD such as Aspire™, Avogreen™, and BioSave™, indicate that the fruit industry is receptive to the new control measures and will implement them if they provide adequate control, are cost effective and compatible with current postharvest practices. Continuous expansion of postharvest biocontrol research worldwide and the related successes, even in the most challenging areas such as control of latent infections creates an optimistic picture for the future of BCPD of fruits.

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Arbuscular Mycorrhizal Fungi in Plant Disease Control

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1 INTRODUCTION

Biological control of plant pathogens presents a compelling method of increasing plant yields by suppressing or destroying pathogens, enhancing the ability of plants to resist pathogens, and/or protecting plants against pathogens. Micro-organisms antagonistic to plant pathogens may be derived from the resident microbial community or may be of foreign origin. Although there are concerns towards the release of an organism of foreign extraction, in general, biological control presents a myriad of benefits such as being a component of the environment, resistant to development of chemical pesticide resistance, being relatively safe and risk free, and by being compatible with sustainable agriculture. Arbuscular mycorrhizal fungi (AMF) form one such group of organisms that can act as bioprotectors of plants. These zygomycetous fungi that form specialized structures such as arbuscules and/or vesicles are obligate biotrophs and utilize host photosynthates for their growth. They are ubiquitous and co-exist with over 80% of terrestrial plants including agricultural or horticultural crops. Their interactions with rhizosphere flora and fauna influence the growth and fitness of the associated plants (Azcon-Aguilar and Barea 1992; Fitter and Sanders 1992). An incompatible association between the host plant and the indigenous AMF community can lead to serious losses in crop yields, indicating the significance of AMF in crop production. In contrast, a compatible association can result in enhanced plant productivity, through enhanced host P nutrition (Ravnskov and Jakobsen 1995), prevention or control of plant diseases caused by soil-borne pathogens (Caron 1989a; St-Arnaud et al. 1995), and/or enhancement of plant hormonal activity (Frankenberger and Arshad 1995).

The rhizosphere, a zone of soil loosely surrounding the roots, is a dynamic environment wherein complex chemical

and microbiological activities occur (Lynch 1990). The mycorrhizosphere is the region of the rhizosphere that is subjected to modifications following AMF colonization of the host plant (Linderman 1988). Induced biochemical changes in the plant as a result of AMF root colonization is collectively termed the “mycorrhizosphere effect.” The mycorrhizosphere effect typically results in a transient or permanent shift in the resident microbial community that may favor the elimination or proliferation of pathogens (Edwards et al. 1998; Meyer and Linderman 1986; Nemec 1994; Paulitz and Linderman 1989). In general, these changes are mediated by modifications in host root membrane permeability that subsequently leads to modifications in root exudate composition (Graham et al. 1981; Ratnayake et al. 1978). Meticulous management of the mycorrhizosphere may serve as an effective, safe, and environmentally friendly alternative to conventional methods of plant disease control.

2 EXAMPLES OF AMF-MEDIATED PLANT DISEASE CONTROL

2.1 Phytopathogenic Fungi

Plant pathogenic fungi contribute significantly to crop damage and yield loss, followed by plant pathogenic bacteria and viruses. The potential of AMF to control various plant pathogenic fungi has been clearly demonstrated (Becker et al. 1999; Bodker et al. 2002; Boyetchko and Tewari 1996; Duchesne et al. 1989; Kapoor et al. 1998; Kasiamdari et al. 2002; Kegler and Gottwald 1998; Krishna and Bagyaraj 1983). In contrast, there are reports wherein AMF inoculation did not have any effect on disease severity (Guillon et al. 2002; Larsen and Bodker 2001; Wyss et al. 1991; Zambolin and Schenck 1983). In order for practical and routine use of

AMF as protectors of plants against plant pathogenic fungi, AMF performance must be consistent, specific, and effective.

Specificity of AMF for the control of crop diseases is crucial in order to mitigate any nontarget effects to beneficial micro-organisms. However, there are conflicting reports on the specificity of AMF. For example, inoculation of micro-propagated banana with *Glomus intraradices* and a *Glomus* spp. isolate reduced rhizome necrosis and external disease symptoms caused by *Fusarium oxysporum* f. sp. *cubense*, but differences between the two AMF isolates were not noted, indicating that either both AMF species were equally effective against the pathogen or that they lacked specificity (Jaizme-Vega et al. 1998). In contrast, eggplant and cucumber seedlings transplanted into soils inoculated with *G. versiforme* and subsequently challenged with *Verticillium dahliae* and *Pseudomonas lacrymans* alleviated wilt symptoms caused by *V. dahliae*, but not *G. mosseae*, *Glomus* spp.-1, or *Glomus* spp.-2, indicating species-specific antagonistic symbiont-pathogen interactions (Li et al. 1997). Pozo et al. (1999) demonstrated the expression of two new basic glucanase isoforms, a phytoalexin elicitor-releasing factor between *G. mosseae* and *G. intraradices* used for the control of *Phytophthora parasitica* var. *nicotianae*. Because of the potential of AMF as bioprotectors against phytopathogens, this is an area that needs further study.

In order to enhance AMF efficacy, some researchers have used an AMF species mixture or a combination of micro-organisms including AMF that act in concert to eliminate pathogens. For example, co-inoculation of groundnut with *G. fasciculatum*, *Gigaspora margarita*, *Acaulospora laevis*, and *Sclerocystis dussii* eliminated the damaging effects of *Sclerotium rolfsii* (Kulkarni et al. 1997). Also, tobacco inoculated with a mixture containing *G. fasciculatum* and *Trichoderma harzianum* effectively controlled damping-off caused by *Pythium aphanidermatum* and black shank disease caused by *P. parasitica* var. *nicotianae* (Sreeramulu et al. 1998). In some cases, microbial mixtures act synergistically with pesticides to result in effective control of plant diseases. A combination of wheat straw, carbendazim, *G. fasciculatum*, and *T. viride* protected safflower seedlings from the root rot pathogen *Macrophomina phaseolina*, resulting in 100% seedling survival (Prashanthi et al. 1997). Sharma et al. (1997) effectively managed ginger yellows disease caused by *F. oxysporum* f. sp. *zingiberi* using a combination of *Gi. margarita*, pine needles, and *T. harzianum*.

The requirement of a fully established AM symbiosis for elicitation of bioprotective activity by AMF has been disputed. The invasion of phytopathogenic fungi is said to be prevented by an aggressively root colonizing AMF species, indicating that AMF root colonization was satisfactory for control of disease. For example, Feldmann and Boyle (1998) found an inverse correlation between *G. etunicatum* root colonization of begonia cultivars and susceptibility to the foliar pathogen caused by the powdery mildew fungus *Erysiphe cichoracearum*. However, it was not clear whether *G. etunicatum* colonization preceded infection by *E. cichoracearum* or whether pathogen suppression was

accompanied by other mechanisms of biocontrol. Using an *in vitro* system, Filion et al. (1999) demonstrated that extracts from the extraradical mycelium of *G. intraradices* reduced the conidial germination of *F. oxysporum* f. sp. *chrysanthemi*. Alternatively, alterations in the chemical equilibrium of the mycorrhizosphere may have resulted in pathogen control. In another study, pea mutants defective for mycorrhization and nodulation challenged with *Aphanomyces euteiches* required a fully established AMF symbiosis for protection against the pathogen (Slezacek et al. 2000).

Several researchers have also demonstrated AMF-mediated reduction of root rot disease in cereal crops (Boyetchko and Tewari 1988; Grey et al. 1989; Rempel and Bernier 1990; Thompson and Wildermuth 1989) and take-all disease of wheat (Graham and Menge 1982). *Phytophthora* spp., which cause diseases in a variety of plants have been model systems for AMF-mediated plant disease control (Cordier et al. 1996; Guillemin et al. 1994; Mark and Cassells 1999; Norman and Hooker 2000; Pozo et al. 1996; Trotta et al. 1996). Using the AMF species *G. intraradices* and pathogen *F. oxysporum* f. sp. *lycopersici* on tomato, Caron and co-workers have shown that the growth medium used (Caron et al. 1985), the application of P (Caron et al. 1986a), and pretreatment of the growth medium with AMF (Caron et al. 1986c) can influence disease severity. Despite proof of AMF potential in controlling plant diseases, few published reports have successfully demonstrated biological control of plant pathogens by AMF in the field (Bodker et al. 2002; Newsham et al. 1995; Torres-Barragan et al. 1996). Newsham et al. (1995) showed that pre-inoculating the annual grass *Vulpia ciliata* var. *ambigua* with an indigenous *Glomus* sp. and re-introducing the grass into a natural grass population extended a favorable effect against an indigenous *F. oxysporum*. Onion pretreated with *Glomus* sp. Zac-19 delayed the development of onion white rot caused by *S. cepivorum* by two weeks in the field and protected onion plants for 11 weeks after transplanting in the field and resulted in a yield increase of 22% (Torres-Barragan et al. 1996). One of the first reports on the effect of indigenous AMF on the development of introduced *A. euteiches* infection and disease development on field-grown pea (Bodker et al. 2002) showed that there was no correlation between AMF root colonization and disease incidence or severity, and emphasized the importance of field evaluations for authenticating the use of AMF as biocontrol agents. Although the indigenous AMF community composition was not described, this study underscores the importance of a richly diverse indigenous AMF community to defend plants from plant pathogens. Thus, there appears to be tremendous potential for AMF control of plant pathogens and the need for more detailed and well-planned and executed studies that will address problems of inconsistent and unreliable results.

2.2 Plant Pathogenic Bacteria

The AMF interact with functionally diverse bacteria such as diazotrophs, biological control agents, and other common

rhizosphere inhabitants (Nemec 1994) that often result in significant alterations to plant growth, yield, and nutrition. Interactions between mycorrhizal fungi and bacteria may have detrimental (Filion et al. 1999; Shalaby and Hanna 1998; 2001) or beneficial effects (Edwards et al. 1998; Gryndler and Hrselova 1998; Li et al. 1997; Ravnskov and Jakobsen 1999), or have no effect at all on the plant pathogenic bacterium (Otto and Winkler 1995).

Glomus mosseae prevented the infection of soybean plants by *P. syringae* (Shalaby and Hanna 1998), by suppressing the population density of the pathogen in soybean rhizosphere. Li et al. (1997) also found that *G. macrocarpum* reduced the infection caused by *P. lacrymans* in eggplant and cucumber, although no positive growth or yield effect was noted, indicating tolerance to the pathogen as a possible mode of action. Inoculation of mulberry with *G. fasciculatum* or *G. mosseae* in combination with 60–90 kg of P per hectare per year reduced the incidence of bacterial blight caused by *P. syringae* pv. *mori* (Sharma 1995). Inoculation of grapevines with AMF reduced the number of fluorescent pseudomonads on the rhizoplane thereby reducing the incidence of grapevine replant disease (Waschkies et al. 1994). Similarly, a reduction in the colonization of apple seedling rootlets by actinomycetes causing replant disease was reported, while a proportionate increase in root colonization by AMF was noted (Otto and Winkler 1995).

2.3 Phytopathogenic Viruses

Viruses remain the least studied amongst all the plant disease-causing target organisms listed for mycorrhizae-mediated biocontrol. The general response of mycorrhizal plants to the presence of viral pathogens is as follows: (a) mycorrhizal plants apparently enhanced the rate of multiplication of viruses in some plants (Daft and Okusanya 1973; Nemec and Myhre 1984), (b) more leaf lesions were found on mycorrhizal plants than on nonmycorrhizal plants (Schönbeck 1978; Schönbeck and Dehne 1979), and (c) the number of AMF spores in the rhizosphere was reduced considerably (Jayaraman et al. 1995; Nemec and Myhre 1984). Enhanced viral multiplication and activity in mycorrhizal plants is speculated to be attributed to higher P levels compared to nonmycorrhizal plants. A similar effect was noted in nonmycorrhizal plants fertilized with P (Daft and Okasanya 1973; Shaul et al. 1999). Some workers found that host plants were more susceptible to AMF colonization following infection by a virus. For example, Schönbeck and Spengler (1979) reported that following the inoculation of mycorrhizal and nonmycorrhizal tobacco (*Nicotiana glutinosa* L.) with tobacco mosaic virus (TMV), mycorrhizal plants exhibited higher levels of AMF colonization. In contrast, mung bean yellow mosaic bigeminivirus reduced the AMF colonization and yield of mycorrhizal plants (Jayaraman et al. 1995), while lack of response to viral infection by a mycorrhizal host was also demonstrated (Takahashi et al. 1994). Early studies using electron

microscopy revealed that mycorrhizae were not viral vectors because virus particles were absent in the AMF hyphae and around arbuscules, suggesting that AMF did not interact with viruses (Jabaji-Hare and Stobbs 1984). Thus, potential for the biocontrol of plant pathogenic viruses using mycorrhizae does not appear to be promising. However, it may be worthwhile to investigate the role of viruses in the reduction of mycorrhizal colonization and related host plant effects.

3 MODES OF MYCORRHIZAE-MEDIATED DISEASE CONTROL

3.1 Host Nutritional Effects

3.1.1 Improved Plant Nutrition

Mycorrhizal plants are generally able to tolerate pathogens and compensate for root damage and photosynthate drain by pathogens (Azcon-Aguilar and Barea 1992; Declerck et al. 2002), because AMF enhance host nutrition and overall plant growth. For example, Declerck et al. (2002) found that *G. proliferum* and a *Glomus* sp. isolate not only stimulated growth and increased shoot P content of banana in the presence and absence of the root rot fungus *Cylindrocladium spathiphylli*, but also reduced root damage by the pathogen, indicating direct interactions between the AMF and the pathogen. In contrast, some reports indicate that AMF are capable of biological control activity (Boyetchko and Tewari 1988; Grey et al. 1989; Rempel and Bernier 1990). It is believed that AMF interact equally with host plants, but in fact AMF prefer one host or host cultivar over another, as shown by Grey et al. (1989) who reported that mycorrhizal barley cultivar W12291 not only exhibited greater control of the barley common root rot pathogen *Bipolaris sorokiniana* than a mycorrhizal cultivar Harmal, but also produced significantly higher yields. On the other hand, biological control activity is dependent on the AMF species as demonstrated for common root rot of barley by Boyetchko and Tewari (1992). There are suggestions that root colonization by natural AMF communities occurring in field soils has an inverse relationship with *B. sorokiniana* infection, indicating not only a direct interaction between the AMF and the pathogen, but also an AMF-mediated improvement in host nutrition (Thompson and Wildermuth 1989). In contrast, there are also reports suggesting a lack of interaction between AMF and *B. sorokiniana* under field conditions (Wani et al. 1991). Interaction between naturally occurring AMF and pathogens or the lack thereof in the field likely depends on the distribution of the organisms particularly under the different crop rotations. Significant reductions in disease severity as a result of AMF colonization and enhanced P uptake followed by modifications in root exudation patterns has also been reported for take-all disease of wheat (Graham and Menge 1982). Improvement in host P nutrition is one of the earliest proposed mechanisms of AMF-mediated pathogen or disease tolerance that is still very pertinent.

3.1.2 Tolerance to Pathogen

Arbuscular fungi are known to enhance plant tolerance to pathogens without excessive yield losses, and in some cases, enhance pathogen inoculum density. This compensation is apparently related to enhanced photosynthetic capacity (Abdalla and Abdel-Fattah 2000; Heike et al. 2001; Karajeh and Al-Raddad 1999) and a delay in senescence caused by the pathogen, which cancels the positive relationship between disease severity and yield loss (Heike et al. 2001). For example, soybean plants grown in the soil infested with *M. phaseolina*, *Rhizoctonia solani*, or *F. solani* exhibited lower shoot and root weight and plant height compared to control plants in soil not infested with the pathogens or with *G. mosseae* (Zambolin and Schenck 1983). The incidence of infection by the pathogens was not affected by *G. mosseae* colonization but the mycorrhizal plants were able to tolerate infection of pathogens better than nonmycorrhizal plants. The efficacy and efficiency of AMF in promoting plant growth enables mycorrhizal plants to tolerate pathogens, as demonstrated by Hwang (1988) using alfalfa challenged with *P. paroecandrum* and Karajeh and Al-Raddad (1999) using olive seedlings. It is unclear whether mycorrhizal alfalfa tolerated *P. paroecandrum* or if other additional mechanisms were involved. Despite the presence of a pathogen benefits of AMF to susceptible hosts can occur until a pathogen inoculum threshold level, beyond which no AMF-mediated benefits can be realized (Stewart and Pflieger 1977). On the other hand, high tissue P levels in mycorrhizal plants may not only improve vigor and fitness of the plant but also modify pathogen dynamics in the mycorrhizosphere by modifying root exudation (Davis and Menge 1980; 1981; Kaye et al. 1984).

Tolerance of the plant to a pathogen can vary depending on the AMF species and their ability for enhancing host nutrition and growth, although some ineffective AMF species reduce pathogen entry by triggering a defense reaction in plants (Davis and Menge 1981). For example, Matsubara et al. (2000) noted that there were significant differences in the ability of *Gi. margarita*, *G. fasciculatum*, *G. mosseae*, and *Glomus* sp. R10 to not only enhance asparagus growth but also in their ability to tolerate the severity of violet root rot caused by *Helicobasidium mompa*. Asparagus seedlings inoculated with *Glomus* sp. R10 had the lowest incidence of violet root rot. This important fact highlights the care that needs to be exercised in the selection of AMF species for biological control of diseases.

3.1.3 Qualitative and Quantitative Alterations in Pathogen Biomass

Modifications in root exudate composition following changes in host root membrane permeability as a result of AMF colonization (Graham et al. 1981) can enforce changes in the rhizosphere microbial equilibrium (Brejda et al. 1998; Edwards et al. 1998; Kaye et al. 1984; Meyer and Linderman 1986). Changes in the rhizosphere microfloral community can

collectively benefit host plants by creating favorable conditions for the proliferation of microflora antagonistic to pathogens such as *Phytophthora* and *Pythium* spp. as shown for eucalyptus seedlings by Malajczuk and McComb (1979). Unfavorable conditions induced by AMF colonization resulted in qualitative changes in the mycorrhizosphere that prevented *P. cinnamoni* sporangial induction in tomato plants (Meyer and Linderman 1986). Proliferation of *G. mosseae* inside grapevine roots was associated with a significant reduction in replant disease-causing fluorescent pseudomonad inoculum in soil (Waschkies et al. 1994). Promoting AMF diversity that will ensure that at least a component of the AMF community may be active against pathogens can further enhance the benefits of this mechanism.

3.2 Competition

The AMF spores in soil are not known to compete for nutrients as spore reserves are utilized for survival until root contact is achieved. Following root entry, competition can occur for infection sites, host photosynthates, and root space (Smith and Read 1997). Competition between AMF and pathogens can be used for physical exclusion of pathogen (Davis and Menge 1980; Hussey and Roncadori 1982; Smith 1988), if the host is preinoculated with AMF. Simultaneous colonization of AMF and the pathogen may not provide a competitive edge for AMF for inoculum build-up (Daniels and Menge 1980) because of its relatively slow growth rate compared with the pathogen. In contrast, some others have noted that competition may not occur between AMF and other organisms (Sempavalan et al. 1995). Competition, as a mechanism of suppressing pathogens by AMF did not receive much consideration, because in some cases pathogens were suppressed even in noncolonized root portions that was later described as induced resistance by AMF (Pozo et al. 1999). In addition, inconsistencies with regard to prerequisites and AMF effects on pathogens have contributed to a lack of interest.

3.3 Physiological and Biochemical Alterations of the Host

Following AMF colonization, host root tissue P levels are typically enhanced which modify the phospholipid composition and therefore the root membrane permeability resulting in a reduction in the leakage of net amount of sugars, carboxylic acids, and aminoacids into the rhizosphere (Graham and Menge 1982; Ratnayake et al. 1978; Schwab et al. 1983). These alterations arrest the chemotactic effect of pathogens to plant roots and discourage pathogen entry. Prior inoculation of maize plants with *G. mosseae* decreased the number of *Alternaria alternata* colony forming units, but when both organisms were inoculated at the same time, there was no effect on pathogen inoculum density in soil (McAllister et al. 1996). It is possible that the *G. mosseae*

symbiont altered membrane permeability of the host roots, thereby reducing the quality and quantity of substances exuded by the roots (Graham et al. 1981), restricting pathogen propagule germination, indicating that the timing of inoculation can enhance biocontrol activity.

3.3.1 Systemic-induced Resistance

Systemic-induced resistance (SIR) is typically the sustained induction of resistance or tolerance to disease in plants by previously inoculating with a pathogen, exposing to an environmental influence or treating with a chemical, which may or may not have antimicrobial activity (Handelsman and Stabb 1996; Kuc 1995). Researchers have suggested that AMF-inoculated plants may employ SIR as a mechanism of biocontrol (Benhamou et al. 1994; Brendan et al. 1996; Trotta et al. 1996). The SIR phenomenon in mycorrhizal plants is demonstrated as localized and systemic resistance to the pathogen (Cordier et al. 1998). An increase in the lignin deposition in plant cell walls following AMF colonization can restrict the spread of pathogens (Dehne and Schönbeck 1979). Using a split root system, Cordier et al. (1998) demonstrated that *G. mosseae* protected tomato plants against *P. parasitica* by reducing pathogen development and spread by increasing cell wall appositions containing callose close to the intercellular hyphae and accumulation of phenolic compounds and plant cell defense responses. Root damage was observed in portions of mycorrhizal root systems not containing mycorrhizal structures. The SIR reaction to the pathogen in mycorrhizal plants was further illustrated by host wall thickenings containing nonesterified pectins and pathogenesis related (PR)-1 protein in the nonmycorrhizal areas of the roots. They also noted that the PR-1 protein was found only in the pathogen-invaded tissues of pea. These responses were observed in the nonmycorrhizal pathogen-infected root tissues that ultimately led to cell death. Bodker et al. (1998) reported that the observed increased resistance to *A. euteiches* in *G. intraradices*-inoculated pea was probably due to an "induced systemic factor," induced by *G. intraradices*. The AMF-mediated SIR phenomenon is speculated to play a role in the protection of potatoes against post-harvest suppression of potato dry rot, wherein dry rot in *G. intraradix*-inoculated potato was reduced by up to 90% compared to uninoculated control (Brendan et al. 1996). This finding suggests that the benefits of AMF inoculation for disease control surpasses the growth and reproduction phase of the host and extends to the storage phase of the product. The area of SIR response in mycorrhizal plants is still developing and several aspects including whether all AMF species can equally elicit a SIR response in the host are not known.

Some researchers have examined the role of PR proteins in the disease control process mediated by AMF (Liu et al. 1995). Enhanced levels of 10 different PR proteins were detected in cotton plants inoculated with *G. mosseae*, *G. versiforme*, or *Scl. sinuosa* challenged with *V. dahliae* compared with plants not challenged by the pathogen. The PR proteins retarded the hyphal growth of *V. dahliae* and killed

their conidia. This appears to be a promising field that can be used for the effective control of plant diseases.

3.3.2 Phytoalexins and Phytoanticipins

Phytoalexins are produced in response to microbial infection (Paxton 1981), whereas phytoanticipins are stored in plant cells in anticipation of or prior to pathogen attack (VanEtten et al. 1995). The level of phytoalexins elicited by pathogens has been shown to be much higher than those elicited by symbiotic organisms (Wyss et al. 1991). The function of an isoflavonoid molecule as a phytoalexin or phytoanticipin can be predicted based on the cellular location of the molecule (Stafford 1992).

An increase in the level of total soluble plant phenolics such as isoflavonoids or flavonoids, lignin, syringic, ferulic or coumaric acids, etc. have been reported as synthesis of phytoalexins following AMF colonization of roots (Harrison and Dixon 1993; Morandi 1989; 1996). Some flavonoids that are not true phytoalexins may also respond to AMF colonization of roots (Harrison and Dixon 1993; Morandi and Le-Quere 1991; Volpin et al. 1995).

The production of phytoalexins as a result of pathogen invasion in mycorrhizal plants has been explored. Tomato plants inoculated with *G. mosseae* posed greater resistance to the pathogen *F. oxysporum* and were found to have increased phenylalanine and β -glucosidase activity and total phenol content in their roots compared to plants inoculated with either organism alone (Dehne and Schonbeck 1979). Sundaresan et al. (1993) reported that a purified ethanol fraction of mycorrhizal cowpea root extract inhibited *F. oxysporum* *in vitro*. However, the isoflavonoid was not identified. Production of phytoalexins in mycorrhizal plants appears to be independent of the effect of fertilizer addition (Caron et al. 1986b). In general, in the presence or absence of pathogens in plant roots, phytoalexins are induced in mycorrhizal plants that neutralize the negative effects of pathogens.

3.3.3 Hydrolases

Differential expression of defense-related genes in mycorrhizal plants has been the recent focus of AMF-mediated biocontrol (Blee and Anderson 1996; Dumas-Gaudot et al. 1996; Lambais and Mehdy 1995; Pozo et al. 2002). Researchers have shown that AMF enter into host (e.g., tomato) roots and induce a local, weak, and transient activation of the host defence mechanism against pathogens such as *P. parasitica*, which involves the induction of hydrolytic enzymes such as chitinase, chitosanase, β -glucanase, and superoxide dismutase (Pozo et al. 2002). In addition, portions of the mycorrhizal root system not containing mycorrhizal structures appear to have alterations in the constitutive isoforms of the enzymes indicating systemic changes following AMF colonization (Pozo et al. 2002). A high positive correlation between the level of glucanase activity in host tissues and pathogen resistance has

been established (Graham and Graham 1991). Further studies examining the role of these glucanases will help in the development of strategies for control of pathogens using AMF.

3.4 Antibiosis

Reports on the production of antimicrobial substances by AMF are not common. However, recently, it was shown that antimicrobial substances (unidentified) produced by the extraradical mycelium of the AMF species *G. intraradices* reduced conidial germination of *F. oxysporum* f. sp. *chrysanthemi*, which was independent of changes in pH (Filion et al. 1999). Budi et al. (1999) isolated a *Paenibacillus* sp. strain from the mycorrhizosphere of *Sorghum bicolor* plants inoculated with *G. mosseae* that exhibited significant antagonism against *P. parasitica*. Regardless of the source of these biocontrol activities, it is important to realize and utilize the significance of AMF in plant disease control. Additional research in this area may prove to be fruitful in the control of pathogenic bacteria and fungi.

4 CHALLENGES AND STRATEGIES TO ENHANCING ARBUSCULAR FUNGAL EFFICACY IN DISEASE CONTROL

4.1 Challenges

Although the efficacy of an AMF species on plant pathogens has been assessed under controlled environments and usually in the absence of other AMF or other organisms (Budi et al. 1999; Kasuya et al. 1996; Li et al. 1997), research indicates that the potential of AMF for control of plant pathogens is high. Limitations in AMF research pertaining to biological control of plant diseases under field conditions are two fold, (a) production of large quantities of AMF inoculum is not feasible because of the obligate biotrophicity status of AMF, and (b) negative interactions between the introduced AMF and the indigenous AMF and microbial community after introduction into field. Challenges posed by interactions between AMF and indigenous microbial community and soil and environmental conditions often determine the success of AMF inoculation in disease control under field conditions. An appreciation of factors that influence AMF efficacy as biological control agents can further enhance their survival, competitiveness and efficacy. For example, it is known that intraradical proliferation of AMF within roots is a host-regulated event (Bever et al. 1996). Therefore, a highly mycotrophic host or host cultivar may be more favorable for AMF proliferation and reproduction than one that is not highly mycotrophic (Feldmann and Boyle 1998; Xavier 1999). In addition, nonconductive soil-environment combinations such as high soil P levels and soil disturbance can affect AMF colonization (Bever et al. 1996; Gazey et al. 1992; Stahl et al. 1988; Stutz and Morton 1996) and efficacy

(Graham et al. 1981; Menge et al. 1978; Ratnayake et al. 1978). Colonization of host roots by AMF is a crucial component in the AMF-mediated SIR response of host plants to plant pathogens as the expression of an SIR response requires a threshold level AMF presence within host roots (Cordier et al. 1996; 1998). The effect of phosphorus on AMF efficacy may be direct, wherein inoptimum P levels impair AMF activity and therefore its ability to effectively control pathogens. In contrast, soil disturbance has an indirect effect where AMF efficacy may be altered by a delay in mycelial network initiation and diversion of carbon for the synthesis or repair of the external mycelial network and not nutrient uptake. Zak et al. (1982) suggested that the ability of AMF to effectively re-establish their mycorrhizal association after disturbance might partially determine their success in a disturbed site. It is not known to what extent soil disturbance affects the biocontrol activities of AMF, but it disrupts the external mycelial network resulting in a severe reduction in mycorrhizal efficacy (Evans and Miller 1990; Stahl et al. 1988).

A richly diverse AMF community ensures qualitatively and quantitatively the presence of AMF species desired for specific activities such as biological control of plant pathogens. However, choice of host genotype and rotation (Bever et al. 1996; Johnson et al. 1992; Talukdar and Germida 1993), levels of fertilizer application (Baltruschat and Dehne 1982; Jasper et al. 1979; McGonigle and Miller 1993; 1996; Vivekanandan and Fixen 1991), tillage (Evans and Miller 1990; McGonigle and Miller 1993; Vivekanandan and Fixen 1991), pesticide application (Manjunath and Bagyaraj 1984; Schreiner and Bethlenfalvay 1997), and the effect of associated micro-organisms (Andrade et al. 1995; Xavier and Germida 2002) are some critical factors that can indirectly alter AMF diversity in soils. For example, continuous cropping selectively enhances the proliferation of parasitic AMF, which are relatively fast growing compared to beneficial AMF, leading to alterations in mycorrhizal biodiversity in the rhizosphere (Johnson et al. 1992). Similarly, one particular AMF host selects from an indigenous AMF pool resulting in the selective enrichment of certain AMF species over others (Xavier 1999). Studies assessing the significance of AMF biodiversity in AMF-mediated biocontrol are rare but critical. Given the importance of AMF to plant health and the complexity of the various microbial interactions, all the relevant factors have to be considered before AMF selected as biocontrol agents can effectively function in the field.

4.2 Strategies

Biological control of plant diseases by AMF under field conditions is the effect of interactions between AMF and various groups of organisms in the rhizosphere. Sikora (1997) proposed "biological system management," a holistic approach for improving plant root systems that adopts

specific cultural practices that promote plant defense mechanisms such as tolerance and/or resistance to pathogens, and the use of organisms that are antagonistic towards pathogens and that target sensitive developmental stages of pathogens. This approach offers a viable alternative to integrated pest management and inundative approaches such as the application of high levels of microbial inocula to the nonrhizosphere soil for biological control purposes, and underscores the significance of mycorrhizae in root health (Sikora 1997).

4.2.1 Enhanced AMF Biodiversity

A diverse AMF community contains a mycorrhizal assemblage and species abundance that naturally aid the host to endure adverse conditions to ultimately enhance plant growth. Research shows that inclusion of host crops (Bever et al. 1996; Johnson et al. 1992) and/or cultivars that exhibit high mycorrhizal responsiveness can significantly improve AMF functioning (Boyetchko and Tewari 1995; Xavier and Germida 1998). Therefore, rotation of crops that are dependent on mycorrhizae will ensure early AMF root colonization and high sporulation of even the most sensitive AMF species in soil. Minimal disturbance to the soil also guarantees early contact between an emerging seedling and the AMF hyphal network in soil that distributes nutrients and initiate early colonization of AMF propagules in soil. Excessive fertilizer and pesticide use can alter plant chemistry and cause changes in AMF assemblage and abundance, resulting in a poor AMF community that does not benefit the host (Gazey et al. 1992; Jasper et al. 1979; Johnson et al. 1992). Caution in the choice of cultural practices that potentially alter AMF diversity would prove to be fruitful.

4.2.2 Improved Understanding of Microbial (AMF) Ecology and Ecosystem Functioning

It is common knowledge that AMF functioning in natural ecosystems can be altered by various factors including interactions with other organisms. However, specifics on the topic are lacking. Knowledge generated from studies addressing AMF efficacy in a typical rhizosphere community, under moisture and salinity stress and soil disturbance, in soils containing extreme indigenous AMF levels, and in the presence of antagonizers is required for the development of effective AMF biocontrol agents.

4.2.3 Selection of Effective AMF

Bagyaraj (1984) suggested that AMF species selection for a desired activity must be based on their ability for survival, aggressive colonization of host roots, and efficacy. Use of AMF species originally isolated from test host roots has proven advantageous for many plant species including agricultural crops, forest tree species, and orchard crops

(Reena and Bagyaraj 1990a,b; Talukdar and Germida 1994; Vinayak and Bagyaraj 1990). Screening procedures must include selection pressure similar to that in which the AMF will be applied.

Research shows that plant pathogens can be controlled not only by the use of biocontrol agents, but also by the induction of resistance responses in plants. Inoculating plants with AMF has been shown to induce resistance in plants. Such plant immunizations are a viable approach for transplant crops because of the ease of AMF inoculation, while more innovative methods are required with direct-sown crops.

4.2.4 Superior Application Technology

Research shows that “priming” plants against pathogens using selective AMF inocula (or plant immunization) helps protect plants by inducing a SIR response (Cordier et al. 1998). The inoculum may be applied to seeds, transplanted crops, or plantlets produced through tissue culture before being transplanted into pathogen-infested fields. Application of the agent prior to transplanting eliminates the need for complex formulations and application techniques, guarantees “targeted placement,” and greater biocontrol activity, reduces costs associated with application and has a minimal impact on the environment (Boyetchko 1996; Glass 1993). Inoculum may include one or more AMF species or other organisms such as bacteria or fungi that exhibit sustained and coordinated biocontrol activity. The application of a multiple agent mixture may concurrently confer control for more than one plant disease by more than one mechanism rather than single inoculants targeted for control of only one plant disease or pathogen. Application of two or more biocontrol agents targeting different life stages of a pathogen may also be more effective than sequential application of the biocontrol agents. In some instances, augmenting soil with organic amendments such as forest humus and charcoal compost has enabled a significant reduction in disease severity (Kobayashi 1993; Wei et al. 1987).

5 CONCLUSIONS

Literature presents a wealth of evidence to indicate potential for AMF-mediated control of plant diseases. Although there are challenges in the form of nonculturability of AMF and therefore mass multiplication for agricultural crops, there is promise for nondirect sown crops, which is currently undervalued and underexploited. The AMF, by increasing crop productivity using existing resources, avoiding resistance development to chemicals, maintaining pollution and risk-free disease control, and conforming to sustainable agricultural practices, offers more than mere plant disease control. In the future, mycorrhizosphere management must become one of the viable and ecosystem friendly solutions to managing plant diseases and reducing pathogen inoculum.

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Commercialization of Arbuscular Mycorrhizal Biofertilizer

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1 INTRODUCTION

Human society today demands the production of high quality food in a most sustainable way causing least damage possible to the environment. Expected benefits include increase in the efficiency of crop production, reduction in agrochemical inputs, and an evaluation of the safety and bioethical aspects in relation to public acceptability. High productivity agriculture exacts a high cost in terms of energy and the environment. Typically, fertilizer and pesticides are used at high levels in the intensive production of plants. More than 150 years of over cultivation with synthetic fertilizers and pesticides has left our soils depleted of the natural biota needed to facilitate the growth of crops. A less costly and nondestructive means of achieving high productivity rests on a establishment of the viable low input farming system. However, to implement such a plan, we must develop plant systems that can efficiently scavenge and utilize soil nutrients present at low levels. The judicious use of nature's own biofertilizers by their biotechnological applications appears to be a suitable answer to this problem. Role of biotechnology in sustainable agriculture can offer a great help towards modern agriculture improvement. In the present review, we have discussed the role of biotechnology, potential biofertilizers with special reference to mycorrhizal biofertilizers, their so far reported synergies, mycorrhizal potential and methodologies for its mass multiplication, different constraints in its commercialization and its future role in achieving sustainable agriculture.

Biotechnology has been defined as the integrated use of biological, physical, and engineering sciences to achieve technological application of biological systems. The goal of

technological application implies a direct relevance to commercialization for mass multiplication of the invented bio-product so as to reach the masses. It is encouraging that both, government and industry are becoming more responsive to natural approaches to growing environmental problems. Individuals and organizations worldwide are coming to realize that excessive use of chemical fertilizers can negatively impact water quality and the environment as a whole. The joint effort, if addressed properly, may lead to a healthy environment for future generations.

Biofertilizers include environment-friendly fertilizers with organisms such as: (a) *Rhizobium* strains for legumes, (b) *Azotobacter* strains for nonlegume crops, (c) VAM strains for use in agriculture, horticulture, and plantation crops, and (d) phosphorus solubilizing bacteria (PSB)—phosphorus dissolving bacteria strains. Most common among these are symbiotic mycorrhiza, *Rhizobium* members and cyanophyceae group, which deliver plant nutrition, disease resistance, and tolerance to adverse soil and climatic conditions. Biofertilizers also known as microbial inoculants may be involved in symbiotic and associative microbial activities with higher plants. They are natural mini-fertilizer factories that are an economical and safer source of plant nutrition and can increase agricultural production and improve soil fertility. They have great potential as a supplementary, renewable, and environment-friendly source of plant nutrients and are important components of any integrated plant nutrient system. Research on biofertilizers has focussed on biological N₂ fixation, plant growth promoting bacteria (PGPR' B) and phosphorus solubilizing microbes (Hegde et al. 1999). Research and development activities involved in this demanding but unexplored field

include microbiological, biochemical, serological, molecular, and ultrastructural techniques, followed by extensive field trials for crop testing before releasing them for agricultural use. During the last decade the phenomenal increase in the production and promotion of biofertilizers in agriculture has been the result of special attention given by the government and interest by entrepreneurs in setting up biofertilizer production facilities. Farmers have also realized the benefits of biofertilizers. For ensuring the rapid growth of biofertilizer usage, constant research support is critical, as it will provide the latest information on improvements of their production technology, applications under different agro-climatic conditions and help in standardizing handling and storage norms. The role of biofertilizers like arbuscular mycorrhizal (AM) fungi in the growth and multiplication of crop plant can prove to be the most effective alternative to fertilizers for enhancing growth and biomass production. Its application has additional benefits like improved vigor and nutrient uptake, disease resistance, and drought tolerance. The economic surplus is used to assess the impact on the overall economic growth and its contribution to economic efficiency and environmental security.

2 MYCORRHIZAL ASSOCIATIONS

Mycorrhizal associations include many taxa of fungi belonging to members of Zygomycetes, Ascomycetes and Basidiomycetes and Deuteromycetes. A characteristic feature of these fungi is that they are generally widespread in soils, exhibit a strong biotrophic dependence on their host plants, and are rarely free living saprophytes. Different types of mycorrhiza are classified into seven different categories on the basis of the extent of root penetration. Among them arbuscular mycorrhizas are the most common and have gained tremendous importance in present day agriculture. The AM symbiosis is the association between fungi of the order Glomales (Zygomycetes) and the roots of terrestrial plants (Harley and Smith 1983). Conservative estimates predict that this ancient symbiosis, dating back to the early Devonian age (398 million years ago), occurs in approximately 90% of the Earth's land plants (Remy et al. 1994). The AM symbiosis is increasingly gaining recognition as an important integral component of natural ecosystems throughout the world. The AM fungus-plant association is a mutually beneficial event: the plant supplies the fungus with carbon (from its fixed photosynthates) while the fungal symbiont assists the plant in phosphate uptake and also converts some unavailable mineral nutrients to available forms for the plant. This bidirectional exchange of nutrients takes place through extensively branched haustoria, commonly called arbuscules. In addition to the nutrient uptake, mycorrhizal fungi improve the performance also of other beneficial microbes, help in resisting root pathogens, and increase the tolerance to extremes of environmental and biological stress. With increased nutritional consumption and a higher uptake of desirable nutrients due to mycorrhization, the biomass, both

above and below the ground, increases. Decomposed biomass, when recycled, improves the soil fertility manifold and this is how mycorrhiza helps in restoring ecosystems.

Above-ground plant development is influenced by below-ground microbial activity. In the presence of mycorrhizal fungi, other micro-organisms such as PSB, many free-living nitrogen-fixing organisms and *Rhizobium* work more efficiently, improving soil fertility and plant growth. Mycorrhizas remain in the soil and form active links with growing plants and mutually benefit each other.

Papers advocating the valuable potential of mycorrhizal inoculations in plant establishments have been published since the 1960s but comprehensive information on their practical exploitation by multiple field trials has not been presented so far (Findlay and Kendle 2001). Immense potential of mycorrhiza has not been so far exploited due to its uncultivable nature unlike other biofertilizers. Mycorrhizas are conventionally propagated using pot-based methods with host trap plants. The disadvantage of this mode is the low recovery of mycorrhizal propagules, contamination by saprobes, pathogens and other mycorrhizal fungi because of improper management techniques and long gaps duration between setup and harvest. Several alternatives to this mode have been designed, but in all current methodologies of cultivating AM fungi, host plant is indispensable. Many variants of these methods have been proposed by various workers to culture glomalean endomycorrhizal fungi, with a bewildering array of claims and counterclaims. These will be described in detail in the present review. All of these involve a plant host, either intact or as root explants. Methodological differences focus mainly on differences in the cultural environment, the most dramatic being the interface between fungus, plant root, and external matrix. The various modes include pot base techniques, hydroponic films (Elmes et al. 1984) or in aeroponic mist chambers (Hung and Sylvia 1988) and the recent *in vitro* root organ culture (ROC) system (Becard and Fortin 1988).

2.1 Pot-Based Techniques

The traditional and most widely used approach has been to grow fungus with a host plant in a solid growth medium consisting of one or a combination of the solid growth media such as soil, sand, peat, vermiculite, perlite, clay, or various types of composted barks. The mycorrhizal inoculum has not been conveniently mass-produced by traditional sand-based pot culture techniques and different micro-organisms frequently contaminate it. In addition, the volume and weight of the inoculum produced by solid growth cultures was sometimes too large and bulky to carry and utilize (Wang and Tschen 1994). Alternative particle size distributions of substrates vary the inoculum production, the ideal substrate proposed for optimum production is proposed to be low in nutrients and carbon (Gaur and Adholeya 2000).

2.2 Aeroponic Culture Techniques

It is a soil-less plant culture system in which nutrient solutions are intermittently or continuously misted onto plant roots. This system allows efficient production of AM fungi, free of a physical substrate. The colonized root material can be sheared, resulting in inocula with very high propagule densities. Furthermore, large quantities of spores can be obtained from the culture system. Aeroponic culture has worked well for several species of *Glomus*. It typically takes 12–15 weeks to obtain an inoculum. There is a 3-week “inoculation phase,” followed by 9 weeks of aeroponic culture for colonized roots or 12 weeks for spore formation. This also has many disadvantages, because the system is also open to other undesirable microbes, which may harbor and propagate along side. Also, the assembly is huge and requires a lot of space; and regular monitoring of the nutrient solution and its flow is required.

2.3 Root Organ Culture Technique

The ROC system is the most attractive cultivation methodology for research; it uses root-inducing transfer-DNA-transformed roots of a host plant to develop the symbiosis on a specific medium *in vitro* (Becard and Fortin 1988). The pathogenic condition known as “hairy root” occurs due to the transfer of root inducing Ri-plasmid from the bacterium *Agrobacterium rhizogenes* (Schenck 1992). These techniques, though challenging have proved useful additions to our knowledge for various aspects of the AM fungal-host symbiosis (Douds 1997). The problem of producing inoculum in bulk is addressed by the ROC of AM isolates, which provides pure, viable, contamination-free inoculum using less space and thus has an edge over the conventional mode of pot-culture multiplication. Pioneering work on *in vitro* cultures was initiated in the early 1960s (Chabot et al. 1992). The use of Ri T-DNA transformed roots of *Daucus carota* as host by *A. rhizogenes* has permitted an increase in spore production of *Glomus mosseae* (Mugnier and Mosse 1987). Only few fungal species have been successfully grown using ROC mode such as *Gigaspora margarita*, *Glomus fasciculatum*, and *G. macrocarpum*, *G. intraradices*, *Gigaspora gigantia*, *G. versiforme*, *G. caledonium*, *G. clarum*, *G. fistulosum*, and *G. etunicatum* (Chabot et al. 1992; Declerck et al. 1996a,b; Declerck et al. 1998; Diop et al. 1992; Douds and Becard 1993; Gryndler et al. 1998; Karandashov et al. 2000; Pawlowska et al. 1999; Souza and Barbara 1999). Though process of bringing them is very difficult, but the list of the new species under *in vitro* is increasing every day. Mass production of spores is prerequisite and mathematical models may be useful as descriptive and predictive tools of sporulation dynamics (Declerck et al. 2001). This mode of culturing AM fungi also provides an opportunity for biochemical and molecular investigations of AM symbiosis, which are otherwise unclear.

Isolation and inoculum production of ectomycorrhiza (EM) and AM fungi present very different problems. Many EM fungi can be cultured on artificial media. Therefore, isolates of EM can be obtained by placing surface-disinfected portions of sporocarps or mycorrhizal short roots on growth medium. The resulting fungal biomass can be used directly as inoculum but, for ease of use, inoculum often consists of the fungal material mixed with a carrier or bulking material such as peat. They can now be produced in a fermentor and by entrapping the mycelium in alginate beads (Le Tacon et al. 1985; Mauperin et al. 1987). Obtaining isolates of AM fungi is more difficult because they will not grow apart from their hosts. Spores can be sieved from soil, surface disinfected, and used to initiate “pot cultures” on a susceptible host plant in sterile soil or an artificial plant-growth medium. Inoculum is typically produced in scaled-up pot cultures. Alternatively, hydroponic or aeroponic culture systems are possible; a benefit of these systems is that plants can be grown without a supporting substrate, allowing colonized roots to be sheared into an inoculum of high propagule number.

3 BENEFITS OF MYCORRHIZAL INOCULATION

The practical application of mycorrhiza in agriculture is relatively new, though its importance has been evident for some 400 million years. The unique advantage of mycorrhizal organisms is that they not only survive in the most stressful environments but also make the plant to do so. The role of mycorrhiza in land reclamation is most recognized these days. Application of mycorrhizal biofertilizer provides a most desirable solution to many such environmental problems. These phosphate-solubilizing biofertilizers are suggested as an alternative or supplements to chemical fertilizers. Some of the benefits offered by mycorrhizal fungi to plants and general soil health improvement are listed below. However, these are not discussed in detail in the present review: (a) *Alleviation of nutrient stress*. Under deficiency conditions, mycorrhizal fungi can increase nutrient uptake. They facilitate the uptake of nutrients such as phosphorus. Difference among arbuscular mycorrhizal fungi (AMF) for plant P acquisition has also been associated with differences in development and function of hyphae like intraradical and extraradical mycelia (Boddington and Dodd 1998; 1999). Many immobile trace elements such as N, S, Ca, Mg, K, Zn, Cu, etc, are also known thus providing better nutrition to the host plant (Clark 1997; Persad-Chinnery and Chinnery 1996). Several reviews are available on the enhanced acquisition of mineral nutrients in plants with mycorrhization (Clark 1997; George et al. 1994; Smith and Read 1997). Turnau et al. (1993) proposed that polyphosphates in the fungal hyphae could sequester metals and minimize transfer to roots of the mycorrhizal plants in stressed conditions, (b) *Enhancement of rooting and reduction of transplant shock*. Mycorrhizal fungi stimulate root production and dramatically increase the volume of soil

the plant can explore. This is especially important on disturbed sites, where nutrients and water availability might otherwise inhibit plant growth, (c) *Alleviation of drought stress*. The increased root volume allows more water to be taken up. Mycorrhizal fungi also enhance the host's osmotic adjusting capabilities, allowing some plants to continue extracting water from soils as they become drier (Ellis et al. 1985; Morte et al. 2000), (d) *Stabilization and aggregation of soil*. Mycorrhizal fungi encircle soil particles and glue larger soil particles together into aggregates. This increases the surface absorbing area of roots 10 to 100 times. They release powerful chemicals in the form of exudates into the soil that dissolve the hard to capture tightly bound soil nutrients. This improves soil structure, producing humic compounds and organic "glues" that bind soils into aggregates and improve soil porosity, increasing air and water movement through the soil while reducing erodibility. Reports are also available suggesting the presence of a protein called Glomalin, which seems to be involved in a very important hypha-mediated mechanism of soil aggregate stabilization (Borie et al. 2000; Degens et al. 1996; Rillig et al. 2002), (e) *Suppression of disease*. Mycorrhizae directly and/or indirectly antagonize disease organisms, increase the number of biocontrol agents around the roots, occupy potential infection sites on the root, and increase host plant vigor to the extent that it can survive disease (Datnoff et al. 1995; St Arnaud et al. 1997; Thomas et al. 1994). They act as biocontrol agent, (f) *Enhancement of nutrient transfer between plants*. This is especially important when nitrogen fixing and non-nitrogen fixing species are planted together. Mycorrhizal roots exploit the soil profile beyond the depletion zone surrounding the absorbing root and its root hairs. Mycorrhizal modifications of the nutrient uptake properties are dependent on the development of extramatrical hyphae in soil, hyphal absorption of phosphate and other micronutrients, their translocation through hyphae over considerable distances and subsequent transfer from fungus to root cells, (g) *Enhancement of beneficial interactions with other microbes*. Mycorrhizal fungi increase the nitrogen made available to the plant by both symbiotic and free-living bacteria. They also increase phosphate uptake to the plant by PSB and support biocontrol agents that are antagonistic to pathogenic organisms, (h) *Salt stress*. The enhancement of mineral acquisition especially that of P, K, Zn, Cu, and Fe due to AMF inoculation is more pronounced under salt-stressed conditions. Studies indicate that AMF-inoculated plants have a greater tolerance to salt stress than un-inoculated plants (Al-Karaki and Hammad 2001; Cantrell and Linderman 2001).

4 AM BIOFERTILIZERS HAVE AN EDGE OVER OTHER BIOFERTILIZERS

This group of biofertilizers is the only among others having fungal system involved. Other biofertilizers exploit bacteria

most commonly. Also this offers wide applicability with a wide range of plants having little selectivity, which is commonly reported in other biofertilizers. Though some exceptions exist with certain nonmycorrhizal families like chenopodiaceae, brassicaceae, and few nonhost plants of nyctaginaceae etc. The storage conditions also are very simple with no extra infrastructural requirements like low temperature and moisture content. Shelf-life is comparatively long. Bacterial systems have short life and cause cell death easily.

The hyphae of fungal system can extend much beyond (a few meters away) the depletion zone and thus can acquire nutrients from a much wider area. The fungal system also produces vegetative structures like chlamydospores and zygospores, which become dormant during periods of environmental stress and germinate with the return of favorable conditions. Thus, they are better equipped for combating unfavorable conditions and have longer shelf-lives compared to bacterial systems. These biofertilizer organisms are broad-spectrum and nonspecific. A single species is known to colonize approximately 90% of land plants. These biofertilizers have broad ecological adaptability and are known to occur in deserts as well as arctic, temperate, tropical, and other habitats. They offer a 25–50% reduction in phosphorus fertilizer application depending on the plant.

4.1 Interaction of Natural Biofertilizers

Different biofertilizers have shown nitrogen-fixing, phosphorus-solubilizing, and phytohormone-producing abilities and are used as for increasing agricultural productivity, for e.g., (*Bradyrhizobium* for legumes (grain, fodder), plant growth promoting bacteria (PGPR) for cereals (wheat, rice, grasses, etc.), *Azolla* for the rice ecosystem, and actinomycetes (*Frankia* spp.) for forest trees. The AM biofertilizer is known to increase the nitrogen-fixing potential of the legumes when given together with *Rhizobium* (Chaturvedi and Kumar 1991) and *Bradyrhizobium* (Werner et al. 1994; Xie et al. 1995). The mycorrhiza first stimulates the nodule bacteria in a sequential process by increasing the tissue phosphorus content; this results in improved nodulation. There are also reports of positive interaction between *Azotobacter/Azospirillum*, and AM fungi (Alnahidh and Gomah 1991). The AM colonization favorably affects the population of these free-living N-fixing bacteria and thus stimulates better growth of plants. The AM colonization also has a stimulatory effect with different nonlegume nitrogen-fixing plant species. In *Casuarina* sp., the double inoculation of AM and *Frankia* improves plant growth and nodulation (Sempavalan et al. 1995).

Two groups of bacteria, chemo-organotrophs like some *Pseudomonas* and *Bacillus* sp., and chemo-lithotrophs, such as *Thiobacillus* sp., are able to solubilize insoluble phosphates.

The AM when given in addition to these bacteria, improve the plant performance. The AM favors the early establishment and efficacy of these bacteria. The synergistic effect of these fungi should thus be exploited on a large scale in the form of biofertilizers to increase the nitrogen-fixing potential of legumes and nonlegume plant species as well as with different phosphate solubilizers. Mycorrhizal fungi interact with a wide assortment of organisms in the rhizosphere. The result can be positive, neutral, or negative on the mycorrhizal association or a particular component of the rhizosphere. For example, specific bacteria stimulate EM formation in conifer nurseries and are called mycorrhization helper bacteria. In certain cases these bacteria eliminate the need for soil fumigation (Garbaye 1994).

5 ENVIRONMENTAL CONCERN ON CHEMICAL FERTILIZER USAGE

Fertilizer production is also an environmental concern. For every ton of phosphoric acid produced, five tons of phosphogypsum are generated. Phosphogypsum is a solid material that results from the reaction of phosphate rock with sulfuric acid. Although it is nearly identical to natural gypsum, it may contain small amounts of sand, phosphate, fluorine, radium, and other elements present in phosphate ore. Federal regulations restrict both use and research involving phosphogypsum because of its radium content and require phosphogypsum to be stacked on the ground. A limited amount of phosphogypsum, with a minimal radium content, is used as an agricultural soil amendment. During the past 50 years, more than 700 MT have accumulated in Florida alone. These enormous stacks, some covering an area of more than 300 hectares and up to 60 m high, have settling ponds on top that contain highly acidic water that can overflow into waterways. New regulations have been enacted to guard against potential spills (Johnson and Traub 1996).

Mycorrhiza offers an alternative to many problems in an ecofriendly, sustainable, and economical way besides creating employment and facilitating poverty reduction. In situations where the native mycorrhizal inoculum potential is low or ineffective, providing appropriate fungi for the plant production system is worth considering. With the current state of technology, inoculation is best for transplanted crops and in areas where soil disturbance has reduced the native inoculum potential. The first step in any inoculation program will be to obtain an isolate that is both infective, and able to penetrate and spread in the root, and effective, or able to enhance the growth and stress tolerance of the host. Individual isolates of mycorrhizal fungi vary widely in these properties, so screening trials are important to select isolates that will perform successfully. Screening under actual cropping conditions is best because indigenous mycorrhizal fungi, pathogens, and soil chemical and physical properties will influence the result.

6 MAJOR CONSTRAINTS AND SOLUTIONS IN COMMERCIALIZATION OF AM BIOFERTILIZER

Perhaps the most important deterrent to the commercial use of mycorrhizal fungi globally is the lack of large-scale multi-location field trials in a variety of agricultural soils and an absence of general lack of awareness among the users. Without such an activity it will be difficult to establish a market for mycorrhizal inoculum. Without a market there is little incentive for a commercial setup to initiate the production of inoculum on a commercial scale, and only large-scale production will make large-scale field trials possible. Other important issues responsible for the general lack of trust amongst the users in its potential are: (a) The lack of cost-benefit analysis to determine the economics of mycorrhizal applications, and (b) The general trend towards excessive fertilization to substitute for the lack of mycorrhizal fungi.

Once large-scale applications of the potential of mycorrhizal inocula are proven to common masses on multi-location fields, lightweight commercial mycorrhizal formulations will need to be developed and new application methods will be devised. Most importantly, from large-scale field tests, cost-benefit analysis will be done accurately to determine the economic benefit derived from the use of mycorrhizal fungi. In the end, this will be the determining factor in the commercial application of mycorrhizal fungi. Biological scientists are rarely able to critically assess the economic factors involved in the application of a new technology. It is important to design the total economic and infrastructure requirement for the setting up a production facility and strict regulatory norms for the quality assessment of the finished product before the release of the product in the market. The involvement of the scientific community is important to define such norms.

The field of applied AM research has suffered for many years from the "chicken-and-egg" syndrome. The inoculum was not widely used because it was not readily available, and it was not available because it was not used. The recent boom in commercial AM inoculants will help break out of the cycle. There have been numerous inquiries about the quality of available inoculum. Unfortunately, only little data on which to base recommendations are available. To remedy this situation, the initiation of a quality control assay (QCA) for commercial AM products is essential which will involve conducting a standard mycorrhizal colonization percentage (MCP) assay on commercial AM inoculum received under before making them available in the market. This will need regular supervision and knowledge of the correct mode of production, formulation, and delivery.

Biofertilizers represent an affordable industry for many developing countries. In many African countries, the use of inorganic fertilizer has increased soil acidity, reducing the yield per ton of fertilizer. Biofertilizers are cheap to manufacture, suitable for small-scale farmers if produced locally (eliminating distribution costs), and the investment in

technology is far lower than that of inorganic fertilizers. Biofertilizers have been produced, packaged, and sold commercially in India, while in a number of African and Latin American countries, biofertilizers have been produced at national research centers. Most importantly, the demand for biofertilizers has outstripped production in almost all these countries. It is estimated that about \$40,000–\$50,000 is required to build a 100–150 MT biofertilizer plant. Alternatively, \$500,000 for 10 plants in different locations could produce up to 1000–1500 MT to meet the demand by rural farmers. With increased production capacity, biofertilizers have a market locally and possibly internationally. Biofertilizers present developing countries with a unique opportunity to enhance their crop yields. Countries like Bangladesh, Brazil, Kenya, Tanzania, Zimbabwe, and Zambia have had successful pilot plants for the production of biofertilizers, and demand has often exceeded production. If any of these countries built a production plant with local and regional markets in mind, they could be exporting their products. India has developed many biofertilizers that are currently on the market for gardeners and farmers. If these products are coupled with crop rotation and irrigation, it is possible to increase crop yields of legumes and cereals. Biopesticides, too, could help increase crop yield, reduce import bills, and increase export earnings. Taken together, they could provide an affordable source of agricultural inputs that would challenge chemical use in rural areas. Chemical fertilizer and pesticide imports and exports from developing countries are low, and production yields are very poor, especially in Africa. Biotechnology will depend on renewable raw materials, and agriculture should play a big role in developing countries' exports.

6.1 Mycorrhizal Commercialization Techniques and Their Formulations

The use of AM fungi in plant biotechnology differs from that of other beneficial soil micro-organisms because the fungi involved are obligate symbionts and therefore recalcitrant to pure culture. Thus specific procedures are required to culture and handle them; specific tools have to be developed and provided to biotechnological producers.

- (a) *Inoculum technology.* Plant inoculation with AM fungi results in the formation of a mycorrhizosphere with selective consequences on other important soil micro-organisms. Therefore the use of AM fungi in plant production needs an appropriate inoculum technology compatible with that used for other beneficial soil micro-organisms. Development of second generation inocula, derived from mixing AM fungi with other inocula, is one such major activity. The use of such inocula will improve plant fitness, and soil aggregation and stability, so increasing yield by biological means. Some of the important issues related to AM biofertilizer commercialization

and its long-term viability in the commercial sector are listed here.

- (b) *Inoculum registration.* Concerns on ecological, biosafety, and bio-ethics demand the requirement for microbial inoculants to be approved and registered. There is need for a centralized government-regulated agency to provide the guidelines for AM fungi-specific standards of inoculum use.
- (c) *Quality control.* Specific protocols for quality control of AM fungal inoculum need to be developed and standardized for application. This is essential not only as a guarantee for producers and users but also for the protection of ecosystems. This would help in quality management and assessment of inoculum potential with every batch of inocula produced. Quality control of commercial AM inoculum is extremely important for developing faith in the user community for its effectively potential. Unless this is achieved, the potential will remain unexplored among the other biofertilizers.
- (d) *Technology transfer.* The product concept for AM fungal inoculum is particularly suitable for industries. Scaling up of production and use of AM fungal inoculum is only economically feasible for them if structures to run concerted field experiments is available. This needs to be offered by researchers working in the area through case studies in the areas of horticulture, fruit production, and revegetation of desertified ecosystems. In India, The Energy and Resources Institute, New Delhi has developed AM mass production technology, which was transferred to two leading industries, however, this is a small move for an agricultural country where economy is important and based on the yield production.

6.2 List of Producers and Formulators of Commercial AM Inoculum

These bio-inoculants are now formulated in different formulations. These are designed on the basis of their application to different crops and locations. They are available in the form of powders, tablets/pellets, gel beads, and balls. Intraradical forms of *Glomus* sp. (vesicles and mycelium fragments) were entrapped in alginate and used as inocula. Isolated intraradical material was found to regenerate in alginate beads and the regenerated mycelium infected roots under controlled conditions (Declerck et al. 1996a,b; Strullu and Plenchette 1991). Glass beads have also been suggested an inoculum type with spores and mycelia inside (Redecker et al. 1995). The application in nursery plantations is normally done using pellets or tablets placed just below the seeds or small plantlets initiating mycorrhization in the hardening phase. Alternative approaches for inoculum disbursement include broadcasting in the field or mycorrhizal products often contain other ingredients designed to increase

Company	Country
AgBio Inc., Westminster	Colorado, USA
Accelerator Horticultural Products	Ohio, USA
AgBio Inc., Westminster	Colorado, USA
Bio-Organics Supply, Camarillo	California, USA
Becker-Underwood, Ames	Iowa, USA
BioScientific, Inc., Avondale	Arizona, USA
EcoLife Corporation, Moorpark	California, USA
First Fruits	Triadelphia, West Virginia
Horticultural Alliance, Inc.	Sarasota, Florida, USA
J.H. Biotech, Inc.	Ventura, California, USA
Mikro-Tek Inc., Timmins	Ontario, Canada
Mycorrhizal Applications, Grants Pass	Oregon, USA
BioGrow TM	North America
Plant Health Care, Inc.	Pennsylvania, USA
MycorTM VAM MiniPlug TM	North America
Premier Horticulture, Red Hill	Pennsylvania, USA
Premier Tech	Québec, Canada
Reforestation Technologies, Salinas	California, USA
Roots Inc., Independence	Montana, USA
T & J Enterprises, Spokane	Washington, USA
TIPCO, Inc., Knoxville	Tennessee, USA
Tree of Life Nursery, San Juan Capistrano	California, USA
Tree Pro, West Lafayette	Indiana, USA
Biological Crop Protection Ltd	Kent, UK
Bio-Organics	Medillin, Columbia
Biorize	Dijon, France
Central Glass Co., Chemicals Section	Tokyo, Japan
Global Horticare,	Lelystad, Netherlands
Idemitsu Kosan Co.	Sodegaura, Chile
MicroBio, Ltd	Royston, Herts, UK
N-Viron Sdn. Bhd	Malaysia
PlantWorks Ltd., Sittingbourne	UK
Triton Umweltschutz GmbH	Bitterfeld, Germany
KCP Sugar and Industries Corporation Ltd	Andhra Pradesh, India
Cadila Pharmaceuticals Ltd	Ahmedabad, India

the effectiveness of the mycorrhizal spores. For example, organic matter is often added to encourage microbial activity, soil structure, and root growth. Stress vitamins improve nutrient uptake and build root biomass. Water absorbing gels help “plaster” beneficial mycorrhizal spores in close proximity to feeder roots and encourage favorable soil moisture conditions for mycorrhizae to form and grow. Organic biostimulants, in general, are effective ingredients in mycorrhizal products. By promoting field competitiveness, stress resistance, and nutrient efficiency, biostimulants reduce barriers to rapid mycorrhizal formation especially during the critical period following root initiation or transplanting. A list of commercially available mycorrhizal inocula is provided in the table above.

Recent advances in the *in vitro* mode of mass multiplication like optimizing various growth parameters

like pH, media manipulations (Douds 2002) can further increase the recovery of propagules. Recent report on the success of co-culturing two different genera together with single host under *in vitro* as it occurs in nature, opens a new scope of an *in vitro* consortium package as inoculum, which may prove more superior in varied edapho-climatic regions where multiple mycorrhizal isolates may function better than single isolate inoculation for future (Tiwari and Adholeya 2002). Industry-based research documentation’s as such are not available to the end users but a recent brief insight into some of the potential techniques by Moutogliss and Beland (2001) along with other alternative production techniques such as bioreactor-based production techniques proposed by Jolicoeur et al. (1999); Jolicoeur and Pirrier (2001) making use of ROC proposes a bright future for AM biofertilizer.

7 CONCLUSIONS

A lot of research done in the past few decades has enabled these fungi to emerge as a potential biofertilizer, a cheap and environment friendly alternative to expensive, harmful chemical fertilizers. This aspect of an alternative to conventional route to more food grain production in a sustainable manner especially gains significance for a developing countries where judicious and large scale utilization of this technology can prove very useful for getting maximum and long-term gains in various wasteland reclamation, reforestation, and afforestation programs apart from giving a much desirable thrust in the production of important agricultural crops. The AM biofertilizer technology can be called poor man's technology. Taking into account the amount of nutrient supplied, biofertilizers are many times cheaper than chemical fertilizers. Biofertilizers improve the quality of produce. They are cheap and economical, the cost benefit ratio is more than 1: 10. It is an ecofriendly practice, improves natural characters of the soil. Uses of biofertilizers maximize ecological benefits and minimize environmental hazards. The demand of biofertilizers is increasing at a tremendous pace, which necessitates the inculcation of the more units to be established in the field to rope of the outgrowing demand potential and the challenges of fabulous future scope. Despite many lacunas in its commercialization and delivery to farmers for exploitation of its potential in agriculture, there is little doubt that AM fungi will emerge as a potential tool for improving crop plants as a promising biofertilizer. Future upgradations in the mode of the AM biofertilizer technology development, redefining the rate-limiting factors and exploration of possible AM combinations along with other potential biofertilizers together as a single package for end users might bring a major boon to agriculture sector using nature's biofertilizers.

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Control of Nematodes by Fungi

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1 INTRODUCTION

Many nematodes are parasites of plants and animals causing severe damage to crops and livestock. Many antihelminthics used to control animal-parasitic nematodes have created resistance in the nematode population, resulting in decreased control efficiency (Nansen 1993). Nematicides, the chemicals which are used to control plant-parasitic nematodes, are often toxic compounds causing both environmental and health problems. Several of these nematicides have been banned in many countries and the current process of phasing out the ozone-depleting substance, methyl bromide, which is an effective nematicide and insecticide, will increase the nematode problem in agriculture (Nordmeyer 1992). Therefore, the possibility to use nematophagous fungi for nonhazardous biological control of parasitic nematodes should be encouraged.

To be able to use nematophagous fungi for biocontrol on a larger scale we need to increase our research efforts to learn more about the life of these organisms, and their interactions with nematodes, plants, and other soil organisms, on ecological as well as cellular and molecular levels. In the present paper we will review and discuss some of the research, which has been performed on nematophagous fungi. Based on recent discoveries we will also speculate on new ways to use nematophagous fungi for nematode control.

2 WHAT ARE NEMATODES?

Nematodes are small roundworms occurring in most environments (soil, aquatic, and marine). Most nematodes are saprotrophs or microbivores in the soil. Other nematodes live part or most of their life cycles as parasites of plants or animals. In the current review we will concentrate on parasitic nematodes, mainly those attacking plants.

2.1 Plant-Parasitic Nematodes

Nearly one hundred species of plant-parasitic nematodes have been described. They affect most crops causing important diseases. As plant pathogens, their effects are time-dependent and usually do not (unlike other pathogens) kill crops, but reduce crop yields to noneconomical levels in spite of “good agricultural” practices (fertilization, irrigation, agrochemicals, etc.).

Plant-parasitic nematodes differ regarding their feeding behavior (Figure 1). Migratory ectoparasites (Figure 1A) remain in the soil and feed on the external cell layers of the root. These nematodes cause little damage. Sedentary ectoparasites remain feeding for long periods at the same place therefore causing severe root damage. Migratory endoparasites enter the root and periodically feed as they migrate through the root. Their damage to root tissue can be very important and may cause other soil pathogens, such as fungi or bacteria, to act synergistically causing complex diseases (Agrios 1997). Sedentary endoparasites (Figure 1B) have developed complex feeding strategies. Essentially, their females become saccate once they start feeding and deeply modify the root tissue in their vicinity to obtain nutrients. The sedentary endoparasites consist of the most important plant-parasitic nematode groups: the root-knot nematodes (*Meloidogyne* spp.) and the cyst nematodes (*Heterodera* and *Globodera* spp.).

2.2 Animal-Parasitic Nematodes

There is a large group of nematodes parasitizing both invertebrates and vertebrates. A number of insect-parasitic nematodes, e.g., *Steinernema* spp., are being developed and commercially used for biological control of plant-parasitic

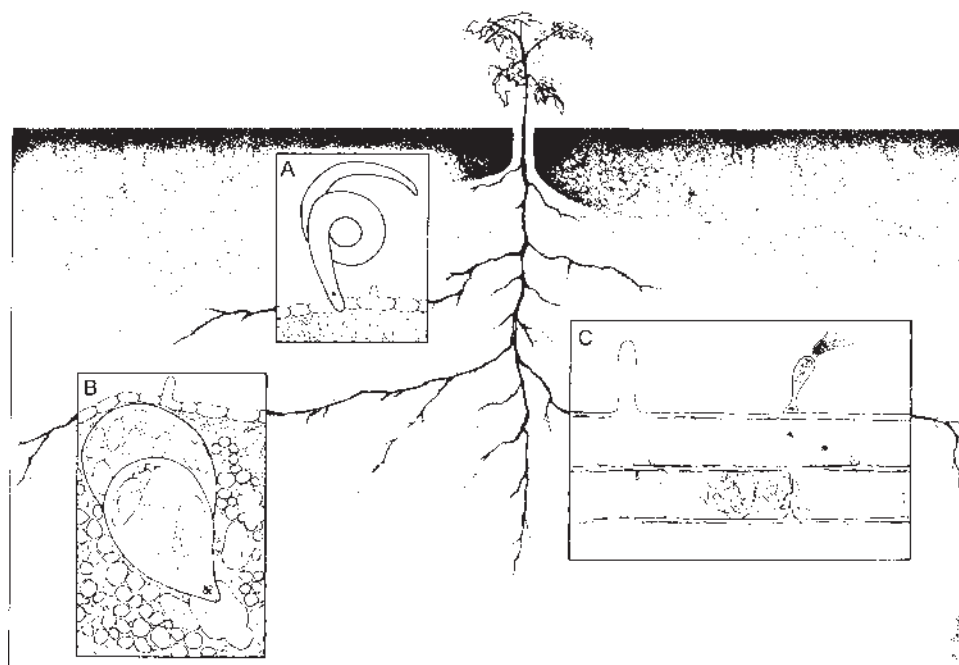


Figure 1 Interactions amongst plant roots, plant-parasitic nematodes, and nematophagous fungi. (A) An ectoparasitic nematode is free living and feeds externally on plant roots by piercing the root surface with its stylet to obtain nutrients. (B) A sedentary endoparasitic nematode (e.g., *Meloidogyne* spp.) performs most of its life cycle inside infected roots. The figure shows a swollen female with an egg sack, feeding on enlarged root cells (giant cells or syncytia). (C) Root colonization by nematophagous fungi. Fungus penetration of root cells sometimes involves formation of appressoria and inter- and intracellular growth in epidermis and cortex.

insects. Other animal-parasitic nematodes, e.g., *Cooperia* spp. and *Ostertagia* spp., cause severe infections and weight loss in animal husbandry (Larsen 2000). Several tropical diseases of humans are also caused by nematodes, such as elephantiasis and river blindness (Poinar 1983).

3 WHAT IS A NEMATOPHAGOUS FUNGUS?

Nematodes constitute a food source for the nematophagous (nematode-destroying) fungi. Living stages of nematodes (eggs, juveniles, vermiform adults, and feeding sedentary females) can be attacked, penetrated, and digested by several types of nematophagous fungi (Jansson and Lopez-Llorca 2001). Dead nematodes (juveniles, vermiform adults, cysts, and root-knot nematode mature females) may also be invaded by nematophagous as well as other (saprotrophic) fungi, but the latter may not be regarded as proper nematode-destroying fungi. For instance, dead vermiform nematodes may be invaded by nematode-trapping fungi, but then the fungi enter the natural openings (mouth, anus, etc.) of the nematodes and never penetrate their cuticles (Nordbring-Hertz and Ståhlhammar-Carléalm 1978), and thus represent the saprophytic growth stage of these fungi. Most

nematophagous fungi are facultative parasites and exist in both saprophytic and parasitic (with infection structures, proper lytic enzyme systems, etc.) stages induced by external and/or internal signals.

The nematophagous fungi are divided into groups depending on their mode of infecting nematodes: nematode-trapping (Figure 2A), endoparasitic (Figure 2B), egg- and female-parasitic (Figure 2C), and toxin-producing fungi (Jansson et al. 1997). Irrespective of the mode of infection, the final result is always the same—a complete digestion of the host. Nematode-trapping fungi capture vermiform nematodes in special trapping organs formed on the hyphae (Barron 1977). The traps can have either an adhesive function where the nematodes stick to the trap, or a mechanical function. These fungi, e.g., *Arthrobotrys oligospora*, are characterized by low host specificity and lower parasitic ability. The endoparasites, e.g., *Drechmeria coniospora*, use their spores to infect the nematode hosts. The spores of these fungi can either be motile, e.g., zoospores, or nonmotile, e.g., conidia of various fungi. The endoparasites have high host specificity and are mostly obligate parasites. The toxin-producing fungi, e.g., *Pleurotus ostreatus*, immobilize their victims using toxins prior to penetration. These three groups of fungi all attack vermiform nematode stages. Females of

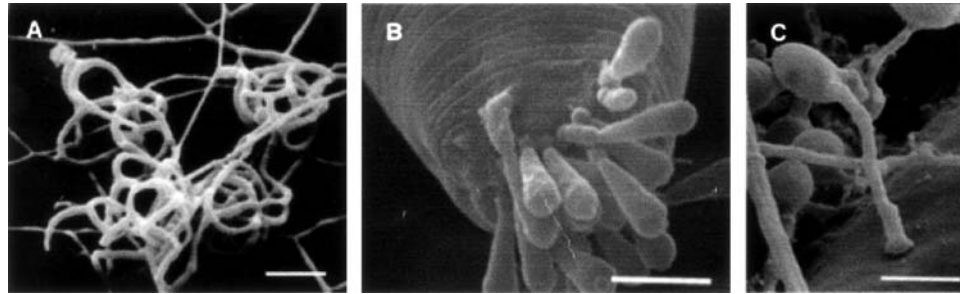


Figure 2 Scanning electron micrographs of nematophagous fungi. (A) Adhesive network trap of *Arthrobotrys oligospora*. Bar = 20 μm . (Courtesy of Birgit Nordbring-Hertz). (B) Adhesive conidia of the endoparasitic fungus *Drechmeria coniospora* adhering to the nematode head. Bar = 5 μm . (From Jansson and Nordbring-Hertz 1983, courtesy of Society of General Microbiology). (C) Protoappressorium formed at the end of a germ-tube of *Pochonia rubescens* on the surface of an egg of *Heterodera schachtii*. Bar = 4 μm . (From Lopez-Llorca and Claughner 1990, courtesy of Pergamon Press).

sedentary endoparasitic nematodes, as well as their eggs, can be infected by facultative egg-parasites, e.g., *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*).

4 HABITAT OF NEMATOPHAGOUS FUNGI

4.1 Soil

The nematophagous fungi are soil inhabitants found in most soil types from tropical to arctic areas of the planet, although they are most frequent in organic soils. One to five species of nematophagous fungi are usually recovered from a soil sample. Their abundance varies from 1.8–150 propagules per gram of soil (Persmark et al. 1996). These variations are due to “environmental factors,” e.g., soil type, organic matter, water content, temperature, and presence of nematode hosts. Since most nematophagous fungi are facultative parasites they also have the capacity to live saprophytically on dead organic matter in soil. Therefore, the soil should obviously be seen as the ultimate sink of nematophagous fungi. The nematophagous fungi, in spite of their saprophytic ability, are not especially good competitors in soil. Therefore, the capacity to invade other organisms—nematodes, other fungi, plant roots—is important for their survival in the soil. The interactions with these potential hosts will be discussed in the following sections.

4.2 Nematodes

By infecting nematodes the nematophagous fungi have the ability to thrive in a sheltered environment protected from other soil microorganisms. There are reports that nematophagous fungi form antibiotic compounds upon infection of nematodes (Barron 1977). The vermiform nematodes are actively moving in soil and are chemically

attracted to the nematode-trapping and endoparasitic fungi (Jansson and Nordbring-Hertz 1988). After contact with the infective structure (trapping organ or spore) the nematode becomes attached, which eventually leads to penetration of the host (Tunlid et al. 1992). Since the nematodes move vigorously and also have high internal hydrostatic pressure the production of an efficient adhesive is vital for successful infection, both as a holdfast and for sealing the penetration area. The nonmotile nematode eggs may be infected by egg-parasitic fungi, the hyphae of which actively grow towards the egg. Upon contact the fungus forms an appressorium that adhere to the eggshell. This stage is followed by penetration of the egg shell and digestion of the contents of the egg (premature or mature juveniles). Infected and dead nematodes may constitute a means for the nematophagous fungi to survive in soil during harsh environmental conditions.

4.3 Fungi

Several species of nematode-trapping fungi, including *A. oligospora*, can attack other fungi, e.g., *Rhizoctonia solani* (Persson et al. 1985). This mycoparasitic behavior takes place by coiling of the hyphae of the nematode-trapping fungi around the host hyphae, which results in disintegration of the host cell cytoplasm without penetration of the host. Although this phenomenon has never been observed in soil it may increase the fitness of the nematode-trapping fungi in soil by reducing competition and providing nutrients. Moreover it may extend the biocontrol capability of nematophagous fungi as biocontrol agents (BCAs) to fungal parasites as well as nematodes. Furthermore, *P. chlamydosporia* has been described infecting propagules of important plant pathogens, such as uredospores of rust fungi (Leinhos and Buchenauer 1992), and oospores of *Phytophthora* and other oomycetes (Sneh et al. 1977).

4.4 Roots

The rhizosphere is a habitat with high microbial activity due to release of nutrients from root exudates and decaying cells. Like other micro-organisms, nematophagous fungi are more frequent in the root zone, with up to 19 times more propagules than in the surrounding nonrhizosphere soil (Persmark and Jansson 1997). Numbers of nematodes are also higher in the rhizosphere than in bulk soil. Since plant-parasitic nematodes attack their plant hosts in the root, understanding of the rhizosphere biology of nematophagous fungi is important in order to use nematophagous fungi for biological control of such nematodes. Nematodes are generally attracted to their host roots by specific or unspecific compounds, e.g., CO₂ (Green 1971). Besides, some nematophagous fungi, e.g., *A. oligospora*, grow chemotropically towards plant roots (Bordallo et al. 2002). Recently, egg-parasitic and nematode-trapping fungi have been found to invade and grow endophytically in epidermal and cortical cells of plant roots (Figure 1C) (Bordallo et al. 2002; Lopez-Llorca 2002b). Root colonization by nematophagous fungi will be further discussed in "Fungi—Root—Interactions."

5 MODE OF ACTION OF NEMATOPHAGOUS FUNGI

The interactions between nematophagous fungi and their hosts involve several steps from recognition (attraction phenomena, contact), production of adhesives and lytic enzymes, differentiation of infection structures, e.g., appressoria and trapping organs, to host penetration and digestion of the host cell contents (Tunlid et al. 1992). These events have been studied using a variety of techniques (microscopic, biochemical, and molecular). In the following section we will discuss some of these interactions in connection with two of the hosts of nematophagous fungi: the nematode and the plant.

5.1 Fungus–Nematode Interactions

5.1.1 Nematode-Trapping Fungi

Arthrobotrys oligospora forms so-called adhesive network traps on which vermiform nematodes are captured. The formation of traps in this fungus is induced chemically by

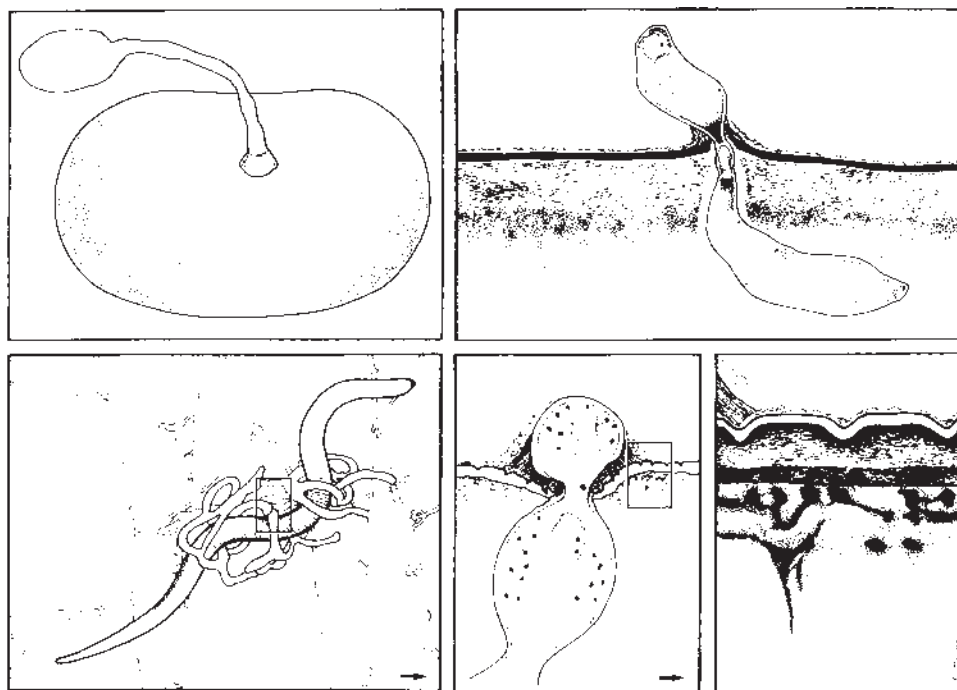


Figure 3 Top figures show infection of nematode egg by the egg parasite *Pochonia* sp. Germling of the fungus forms an appressorium after contact with the egg surface (left). An adhesive is formed and the fungus penetrates the egg shell, grows inside the egg, and digests its contents. Bottom figures illustrates capture and infection of a nematode by the nematode-trapping fungus *Arthrobotrys* sp. A nematode is captured in the three-dimensional network trap (left). The middle figure (enlargement of left figure) shows the trap, covered with adhesive, penetration of the nematode cuticle, and formation of an infection bulb. The trap and infection bulb contain dense bodies (dark dots). The right figure shows an enlargement of the middle figure with the multilayered nematode cuticle covered with a surface coat.

small peptides, e.g., phenylalanyl valine (Nordbring-Hertz 1973) or by nematodes (Nordbring-Hertz 1977). The presence of traps is a prerequisite for infection of living nematodes, and in fact increases the ability of the fungus to chemically attract nematodes (Jansson 1982). After contact between the fungal trap and the nematode cuticle (Figure 3 bottom left) a possible contact recognition step occurs involving a fungal lectin binding to *N*-acetylgalactose amine (Gal-NAC) on the nematode surface (Nordbring-Hertz and Mattiasson 1979). The nematode surface, the cuticle (Figure 3 bottom right), consists of several layers containing proteins (mainly collagen), lipids, and carbohydrates (Bird and Bird 1991). Externally to the cuticle a surface coat (or glycocalyx) consisting of glycoproteins is found (Bird and Bird 1991). The surface coat is probably the part of the nematode surface most relevant to recognition and adhesion of nematophagous fungi, since proteolytic removal of this structure results in reduced adhesion of bacteria and spores of endoparasitic nematophagous fungi (Bird 1985; Jansson 1993).

The trapping organ of *A. oligospora* contains an adhesive material. Upon contact with the nematode surface, a recognition step possibly mediated, at least partly, by lectin

binding, induces changes in the structure of the adhesive leading to capture of the nematode (Veenhuis et al. 1985). The adhesive undergoes changes from an amorphous material to a fibrillar structure, more organized with fibrils perpendicular to the nematode surface. This may anchor the nematode to the trap thus facilitating infection. In contrast, the adhesive of the endoparasitic fungus *D. coniospora* does not appear to change and has a fibrillar structure even in the absence of nematodes (Jansson and Nordbring-Hertz 1988). After the firm attachment to the host surface, *A. oligospora* penetrates the nematode cuticle and forms an infection bulb (Figure 3 bottom middle), from which trophic hyphae grow out to digest the nematode contents (Veenhuis et al. 1985). In the trap and the infection bulb organelles, so-called dense bodies, now appear. These organelles are not present in ordinary hyphae and have been suggested to contain hydrolytic enzymes used for penetration of the cuticle and digestion of host (Jansson and Nordbring-Hertz 1988; Veenhuis et al. 1985).

As in many other instances of fungal penetration of their hosts' surfaces, nematophagous fungi appear to use both enzymatic and physical means. The nematode cuticle mainly

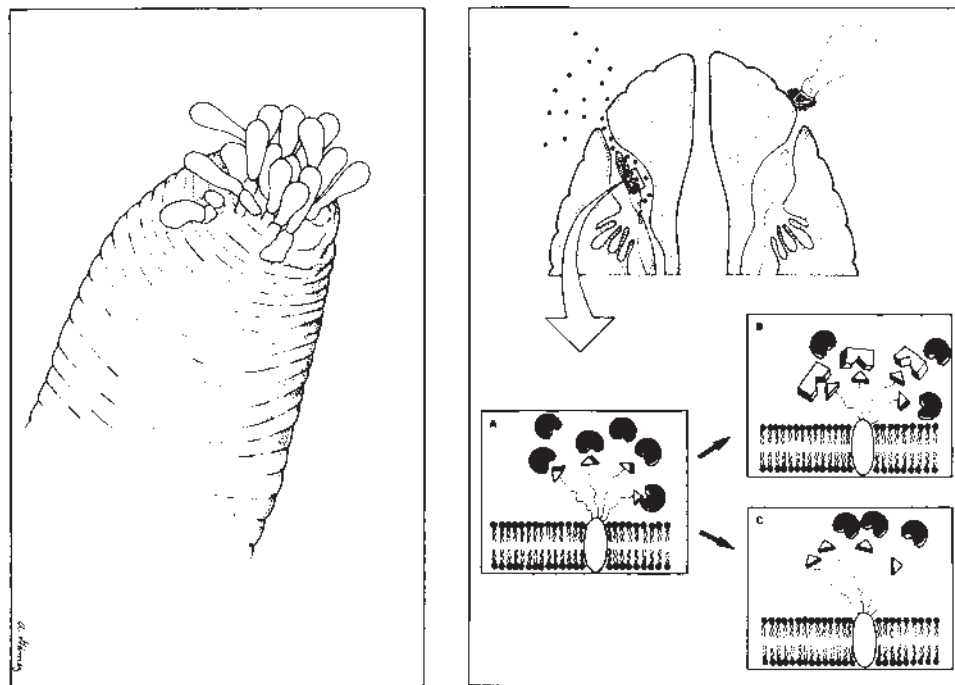


Figure 4 Hypothesis of interference with nematode chemotaxis. Conidia of the endoparasitic nematophagous fungus *Drechmeria coniospora* adhere to the sensory organs at the anterior end of a nematode and block nematode attraction (left). The top right figure illustrates the tip of the nematode head with two amphids (chemosensory organs). The chemotactic factors (black dots) are transported to the neuron membranes where the chemoreceptors are located. (A) shows a proteinaceous chemoreceptor with carbohydrate chains, where the terminal sugar (purportedly mannose or sialic acid, triangle) binds to a chemotactic factor leading to normal attraction of the nematode. (B) Illustrates blocking of terminal carbohydrates with lectins (Con A and Limulin) thereby inhibiting nematode chemotaxis. (C) Shows enzyme (mannosidase and sialidase) obliteration of terminal sugar moieties leading to inhibition of chemotactic behavior. Proteolytic enzymes, hydrolyzing the chemoreceptor (membrane protein), have a similar effect.

contains proteins (Bird and Bird 1991) and therefore the action of proteolytic enzymes may be important for penetration. A serine protease, PII, from *A. oligospora*, has been characterized, cloned, and sequenced (Åhman et al. 1996). The expression of PII is increased by the presence of proteins, including nematode cuticles (Åhman et al. 1996). PII belongs to the subtilisin family and has a molecular mass of 32 kDa (for review see Jansson et al. 1997). Serine proteases will also be discussed in "Egg Parasites." During decomposition of infected nematodes *A. oligospora* produces a lectin (*A. oligospora* lectin, AOL), which functions as a storage protein within the nematode host, and may constitute as much as 50% of the total fungal protein. AOL is a multispecific lectin, which binds to sugar chains common in animal glycoproteins, including those of nematodes (Rosén et al. 1997).

5.1.2 Egg Parasites

Egg-destroying fungi act on nematode eggs at two levels: directly as true parasites by penetrating and infecting eggs, and indirectly by causing distortions in the larvae or embryos they contain (Morgan-Jones and Rodrigues-Kábana 1988). The former mode of action is well documented and is largely responsible for cases of soil suppressiveness to nematodes. In this chapter we will describe the direct mode of fungal infection of nematode eggs mainly using the egg-parasitic fungi *Pochonia rubescens* (syn. *Verticillium suchlasporium*) and *P. chlamydosporia* (syn. *V. chlamydosporium*).

Upon growth of germ tubes the hyphal tips swell and differentiate into appressoria at contact with nematode eggs (Lopez-Llorca and Claugher 1990) (Figures 2 and 4), as well as on artificial, especially hydrophobic surfaces (Lopez-Llorca et al. 2002b). An extracellular material (ECM) probably functions as adhesive, but possibly also seals the hole caused by the penetration hypha (Figure 3, top). This ECM can be labeled with the lectin Concanavalin A, indicating that the ECM contains mannose/glucose moieties probably on side chains of glycoproteins (Lopez-Llorca et al. 2002b). Most ECMs of fungal hyphae consist of proteins and carbohydrates (Nicholson 1996).

Early evidence of eggshell penetration by *P. chlamydosporia* came from studies of plant- and animal-parasitic nematode egg infection (Lysek and Krajci 1987; Morgan-Jones et al. 1983). In eggshells, low electron dense areas were found in the vicinity of penetration hyphae of *P. rubescens* on cyst nematodes (Lopez-Llorca and Robertson 1992a) suggesting involvement of eggshell degradation enzymes. Nematode eggshells mostly contain protein and chitin (Clarke et al. 1967) organized in a microfibrillar and amorphous structure (Wharton 1980). Therefore, a search for extracellular enzymes degrading those polymers was carried out. A 32-kDa serine protease (P32) was first purified and characterized from the egg parasite *P. rubescens* (Lopez-Llorca 1990). Involvement of the enzyme in pathogenesis was suggested by quick *in vitro* degradation of *Globodera pallida* egg shell proteins (Lopez-Llorca 1990), but most of all by its

immunolocalization in appressoria of the fungus infecting *Heterodera schachtii* eggs (Lopez-Llorca and Robertson 1992b). Although pathogenesis is a complex process involving many factors, inhibition of P32 with chemicals and polyclonal antibodies reduced egg infection and penetration (Lopez-Llorca et al. 2002b). The similar species *P. chlamydosporia* also produces extracellular proteases (VcP1) (Segers et al. 1994), which are immunologically related to P32 and similar enzymes from entomopathogenic fungi (Segers et al. 1995). VcP1-treated eggs were more infected than untreated eggs suggesting a role of the enzyme in eggshell penetration by egg-parasitic fungi. Recently, several chitinolytic enzymes of *Pochonia* spp. were detected. One of those accounting for most of the activity was a 43-kDa endochitinase (CHI43) (Tikhonov et al. 2002). When *G. pallida* eggs were treated with both P32 and CHI43, damage to eggshell was more extensive than with each enzyme alone, suggesting a co-operative effect of both enzymes to degrade egg shells (Tikhonov et al. 2002).

5.1.3 Interference with Nematode Chemoreception

Carbohydrates present on nematodes are not only involved in the recognition step of lectin binding, but also appear to be involved in nematode chemotaxis (Jansson 1987; Zuckerman and Jansson 1984). The main nematode sensory organs are the amphids and the inner labial papillae located around the mouth in the head region of the nematodes (Ward et al. 1975). Figure 4 (right) schematically depicts the structure of amphids in the nematode head. The chemoreceptors are thought to be located on the neuron membrane. There are 28 neurons in the nematode *Caenorhabditis elegans*, each with a passage to the environment, but the number of receptors is not known (Ward 1978).

A hypothesis of the involvement of carbohydrates in nematode chemoreception was put forward by Zuckerman (1983); Zuckerman and Jansson (1984). The chemoreceptors, purportedly glycoproteins, could be blocked (Figure 4B) by lectins (Concanavalin A binding to mannose/glucose residues, and Limulin binding to sialic acid) resulting in loss of chemotactic behavior of bacterial-feeding nematodes to bacterial exudates (Jeyaprakash et al. 1985). Furthermore, treatment of the nematodes with enzymes (mannosidase and sialidase) thus obliterating the terminal carbohydrates (Figure 4C) also resulted in decreased chemotactic behavior (Jansson et al. 1984), demonstrating the importance of these carbohydrate moieties in nematode chemotaxis.

Interfering with nematode chemotaxis, thereby inhibiting their host-finding behavior, may be a possible way of controlling plant-parasitic nematodes. In a pot experiment using tomato as host plant and *Meloidogyne incognita* as parasitic nematode, addition of Concanavalin A and *Limax flavus* agglutinin (sialic acid specific lectin) resulted in decreased plant damage by the nematode compared to controls (Marban-Mendoza et al. 1987). Addition of lectins (or enzymes) on a field is not feasible, but the possibility to use, for instance, lectin-producing leguminous plants have

been shown to reduce galling by root-knot nematodes (Marban-Mendoza et al. 1992).

The endoparasitic nematophagous fungus *D. coniospora* infects nematodes with its conidia, which adhere to the chemosensory organs (Figures 2 and 4) (Jansson and Nordbring-Hertz 1983). Conidial adhesion was suggested to involve a sialic acid-like carbohydrate since treatment of nematodes with the lectin Limulin, and treatment of the spores with sialic acid, decreased adhesion (Jansson and Nordbring-Hertz 1984). Furthermore, nematodes with newly adhered spores lost their ability to respond chemotactically to all attraction sources tested, i.e., conidia, hyphae, and bacteria, indicating a connection between adhesion and chemotaxis through carbohydrates on the nematode surface (Jansson and Nordbring-Hertz 1983). The conidia of *D. coniospora* adhere to the chemosensory organs of *Meloidogyne* spp., but do not penetrate and infect the nematodes. Irrespective of the lack of infection the fungus was capable of reducing root galling in tomato in a biocontrol experiment (Jansson et al. 1985), again indicating the involvement of chemotactic interference.

5.2 Fungi–Root–Nematode Interactions

Nematophagous fungi can infect, kill, and digest living nematodes. Most of these fungi can also live saprophytically and some even have mycoparasitic abilities. Since most plant-parasitic nematodes attack plant roots, the rhizosphere biology of nematophagous fungi is important from a biological control point of view. We previously described that nematophagous fungi were more abundant in the rhizosphere than in bulk soil (Persmark and Jansson 1997). In recent investigations we studied the colonization of internal cells of plant roots (Bordallo et al. 2002; Lopez-Llorca et al. 2002a). In these experiments we used axenic barley and tomato plants grown in vermiculite and inoculated with the nematode-trapping fungus *A. oligospora* or the egg-parasite *P. chlamydosporia*. Roots were sequentially sampled, cryo-sectioned, and observed under light- or cryo-scanning electron microscopes. Both fungi grew inter- and intracellularly, formed appressoria when penetrating plant cell walls of epidermis and cortex cells, but never entered vascular tissues (Figure 2C). In contrast to *Pochonia* spp., appressoria had never been observed previously in *A. oligospora*. Using histochemical stains we could show plant defense reactions, e.g., papillae, lignitubers, and other cell wall appositions induced by nematophagous fungi, but these never prevented root colonization. Callose depositions in papillae are shown in Figure 5. Nematophagous fungi grew extensively especially in monocotyledon plants producing abundant mycelia, conidia, and chlamydospores (*P. chlamydosporia*). Necrotic areas of the roots were observed at initial stages of colonization by *A. oligospora*, but were never seen at later stages even when the fungi proliferated in epidermal and cortical cells. *P. chlamydosporia* colonized roots displayed higher proteolytic activity than uninoculated control roots

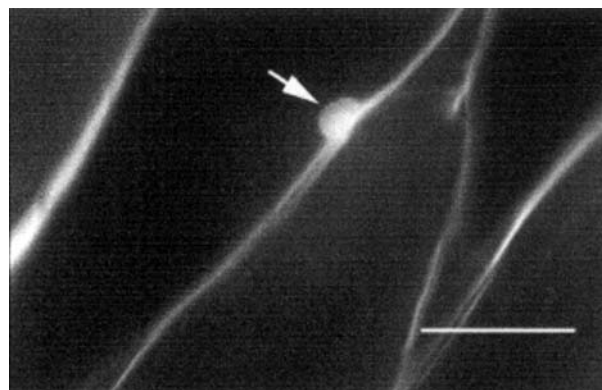


Figure 5 *Arthrobotrys oligospora* colonizing barley root. Callose deposition in cell wall papillae (arrow) stained with Sirofluor. Bar = 20 μ m. (From Bordallo et al. 2002, courtesy of Blackwell Science).

(Montfort et al. unpublished). The significance of this fact for biological control is under investigation in our laboratory.

The growth of the two nematophagous fungi in plant roots appears to be like that of an endophyte. Whether this endophytic growth induces systemic resistance to nematodes and/or plant pathogens in plants is yet unknown, but worth further investigations. Endophytic rhizobacteria reducing plant-parasitic nematodes have been described (Hallmann et al. 2001), as well as the reduction of root-knot nematodes by arbuscular mycorrhizal fungi (Waecke et al. 2001). If this is the case also in nematophagous fungi this will open up a new area of biocontrol using these fungi. The internal root colonization by egg-parasitic fungi, e.g., *Pochonia* spp., may give the fungi an opportunity to infect nematode eggs in egg sacks of root-knot nematodes inside the roots and reduce subsequent spread and infection of roots by the second generation of juveniles. Structures resembling trapping organs were observed in epidermal cells colonized by *A. oligospora*, and these may serve the purpose of trapping newly hatched juveniles escaping the roots. The ability to colonize plant roots may also be a survival strategy of these fungi and could explain soil suppressiveness to plant-parasitic nematodes in nature. The colonization of plant roots is a new area of research that deserves in-depth investigations, not the least for biocontrol purposes. This is presently underway in our laboratory.

6 BIOLOGICAL CONTROL OF PLANT-PARASITIC NEMATODES

The use of nematophagous fungi for biological control of plant-parasitic nematodes has a long history (for extensive review on earlier experiments see Stirling 1991). The investigations have resulted in variable and confusing results—sometimes excellent control results, at other

occasions no control at all. The reasons for these varying results may be many, but the major reason is probably lack of knowledge, both on physiology and ecology of the nematophagous fungi. Their interaction in soil with nematodes, plants, other organisms, and the soil environment is little understood because of difficulties of working in the complex soil matrix and also lack of good methods for these purposes. We believe it is necessary to perform both laboratory experiments and field studies using old and new techniques to understand the complexity of nematophagous fungi interactions with their environment. In the following section we will discuss some of the possibilities to use nematophagous fungi for biological control.

6.1 Sources of Antagonists to Nematodes

Just as the biodiversity of the planet is becoming more and more reduced, scientists and the general public are becoming more aware of its importance in “practical” terms. Microbial biodiversity is little known but even the most “humble” soil may contain strains of microorganisms with interesting antagonistic properties. In the past, the use of antagonists for biocontrol purposes was restricted to the culturability of the putative organisms detected. Nowadays, in the post-genomic era, our capabilities of handling genetic material of organisms allow the detection of microorganisms or their metabolites in the environment. The classical approach for finding antagonists has been to search for them in places where their target did not cause damage or disease in spite of host presence. Clear examples of these places are suppressive soils.

The most studied sources for fungal antagonists of nematodes are soils naturally suppressive to plant-parasitic nematodes. The first example to be discovered was the decline of *Heterodera avenae* populations in cereal monocultures. Using previous data from nematode population dynamics and with a stepwise approach, Kerry (1987) and co-workers found fungal parasites of females (*Nematophthora gynophila*) and eggs (*P. chlamydosporia*) to be the biotic causes for nematode suppression. Similar situations have been found elsewhere in the world with soils suppressive to cyst nematodes. Root-knot nematodes have a much wider host range than cyst nematodes. Besides, many crop hosts of root knot nematodes are grown under intensive agricultural conditions with regular use of agrochemicals. Under these conditions soil antagonists are not favoured. Therefore, fewer examples of natural suppressive soils to root-knot nematodes have been found. Among these are tree orchards in the United States, where the egg-parasitic nematophagous fungus *Dactylella oviparasitica* was first isolated (Stirling and Mankau 1978). In a similar environment, ring nematodes (*Criconebella* spp.) were found to be suppressed by *Hirsutella rhossiliensis*, an endoparasitic nematophagous fungus (Jaffee and Zehr 1982). Both antagonists are examples of organisms with an important ecological role but difficult to use biotechnologically. Both fungi have a limited growth rate and sporulation. Soil

suppressiveness to nematodes is a very complex phenomenon. In a study to find causes for nematode decline, Persmark et al. (1995) did not find differences in nematophagous fungi populations between putative suppressive and conducive soils in Central America.

Searching for nematophagous fungi in agroecosystem soils have yielded fungal antagonists with potential as BCAs of plant-parasitic nematodes. For instance, cyst nematodes were infected by the entomopathogenic species *Lecanicillium lecanii* (syn. *Verticillium lecanii*) in the United States (Meyer et al. 1990). In other instances, fungal antagonists have remained undescribed and their use halted for lack of sporulation, e.g., ARF-18, a sterile fungus infecting cyst nematodes in the United States (Kim and Riggs 1991). The search for nematophagous fungi in natural “undisturbed” ecosystems is another approach for finding antagonistic potential, e.g., the Antarctic (Gray 1982) or the tropical rain forest. Costa Rica still offers protected areas where such studies can be carried out. Our laboratory is presently involved in such a survey.

Another approach of using fungi for nematode control is to identify nematophagous metabolites. These substances can be identified from nonpreviously described nematophagous fungi, e.g., cuticular disruption of *Caenorhabditis elegans* by *Byssoschlamys nivea* (Park et al. 2001). A search for nematocidal molecules from *Arthrobotrys* and other fungi was carried out by Anke et al. (1995). In the future, genes (e.g., toxins, enzymes) can be a source of antagonistic potential against nematodes, which can be used for improvement of BCAs.

6.2 Fungal Biocontrol Delivery Systems for Nematodes

After isolation, antagonists of nematodes have to be developed for biocontrol. The first step usually involves a screening program including traits relevant to the performance of these organisms in the field. Root colonization is considered as an important ability for biocontrol of plant-parasitic nematodes (Bourne et al. 1996). Evaluation of pathogenicity, especially to nematode eggs, is difficult since these stages are nonmotile. Several techniques have been used for this purpose (Gunasekera et al. 2000; Irving and Kerry 1986; Lopez-Llorca et al. 2002b). Once fungal antagonists have been rated, promising strains should be stored in a safe way. To this respect, an assessment of the existing methods of fungal preservation on nematophagous fungi should be carried out.

Stirling and colleagues developed formulations of nematophagous fungi for use in laboratory (Stirling et al. 1998a,b) and field experiments (Stirling and Smith 1998). Control of root-knot nematodes was achieved in microcosms, but not in the field. The authors stressed that formulations with greater activity should be developed for field experiments. Other factors than formulation design could also be responsible

for poor performance of BCAs in the field. Soil microfauna, e.g., enchytraeids (Jaffee 1999) and nematodes, have been found to consume the nematophagous fungi inoculum. Soil receptivity to inoculum of nematophagous fungi should be assessed before delivery of large amounts of inoculations are to be carried out (Wakelin et al. 1999).

The effects of nontarget organisms beneficial to the crop rhizosphere, e.g., mycorrhizal fungi and rhizobacteria, on nematophagous fungi have not yet been characterized. Such studies would improve the design of synergistic methods of approaching biocontrol of root diseases caused by nematodes and other pathogens.

7 CONCLUSIONS

To use nematophagous fungi for biological control of nematophagous fungi we need to know much more about their biology, on physiological as well as ecological levels. This is especially true for the tri-trophic interactions between the fungi, nematodes, and plant roots. While the invasion of roots by plant-parasitic nematodes is fairly well established, little is known about root colonization by nematophagous fungi. The endophytic growth of these fungi needs to be further investigated using cellular, molecular, as well as ecological studies, to find out if the nematophagous fungi can induce, for instance, systemic resistance in plants to nematodes and other plant pathogens. If this is true and can be developed further, it will add a new property of nematophagous fungi apart from their nematode-destroying ability, and selection of nematophagous fungi for their root colonizing capacity may have equal importance to their nematophagous ability.

Combinations of different types of nematophagous fungi, e.g., egg-parasitic and nematode-trapping fungi, which infect various stages of the nematode life cycles, have been rarely studied in biocontrol experiments. Good egg parasites will be able to destroy nematode eggs, even in root-knot nematode galls inside roots, and nematode trappers may capture juveniles entering or leaving roots. Such experiments need to be conducted in both laboratory and field investigations for development of BCAs as a practical way to control plant-parasitic nematodes.

In the soil and root environment, nematophagous fungi are difficult to study mainly due to lack of appropriate methods. New techniques therefore have been developed and used, especially for tracing fungi in the environment. Antibodies from spore or hyphal surfaces have been tried, but these usually show cross reactions between species and are difficult to use. Molecular techniques probably need to be employed. These could involve, for instance, transformation with marker genes [e.g., the green fluorescence protein (GFP)], or development of molecular beacons or similar approaches based on specific DNA sequences.

Development of proper delivery systems (production, formulation, application, etc.) and the effect on nontarget organisms are key factors involved in the good performance

of nematophagous fungi as BCAs. A fair deal of basic research should be invested in these fields. These efforts will also find applications in other fields of root health.

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Fungi in Food Technology: An Overview

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1 INTRODUCTION

The concepts and practical applications of fungal biotechnology continue to make significant contributions to food science. Several recent books and monographs have been dedicated to this topic (Hui and Khachatourians 1995; Khachatourians and Arora 2001; Khachatourians and Arora 2002; Khachatourians et al. 2002; Pointing and Hyde 2001; Rajak 2000; Singh and Aneja 1999). Table 1 shows a few key food products and processes that depend on applied mycology and biotechnology. Without exception many aspects of food ingredient and process technologies involving fungi are impacted by research in biotechnology, for example, functional foods, nutraceuticals, value added foods, and food pathogen and safety detection systems. Inevitably, and beyond the laboratory level research, the development of food biotechnology depends on research and developments in engineering and down stream processing. It is in this context that the convergence of food science and engineering create crosscutting opportunities for the two disciplines, biotechnology and engineering. An obvious group of foods in this crosscut is the large-scale production of fermented foods, edible mushrooms, so called, single cell proteins (SCP), and fermented beverages. Finally, fungi in food technology have benefited from intellectual property rights associated with both engineered fungi and process patents. Fungal biotechnology should enjoy being a primary driver of world food production technologies.

2 CURRENT FOOD SUPPLY AND DEMAND

With the geometric doubling of population and marginally arithmetic doubling of food production, the ideas of Robert

Malthus proposed in 1798 remains as controversial as when they first appeared. Malthus's hypothesis remained an important unanswered question for years after the bicentennial of Malthus's paper. Brown et al. (1999) point out what additional issues complicate the simplistic Malthusian thought are additional constraints of public health and global economy. One major intervening force has been the emergence of new and reemerging infectious diseases, which in the absence of global war have had an equivalent effect in terms of human suffering and death worldwide. Ironically, these epidemics are occurring again in spite of advanced medical technologies and accelerated methods of health care delivery and immunization. To further complicate the issue, the conventional practice of agriculture has not doubled and cannot double the production of plant and animal-based foods, which are still at the mercy of nature and its disasters. Presumably, biotechnology has emerged as a solution, or an option for one, at least for the moment.

Disciplinary crossover of biochemistry, genetics, microbiology, nutritional sciences, engineering, and emergence of biotechnology set the stage for reconsidering the paradigm of agriculture from traditional breeding for food plants (Khachatourians 2002). The strongest impact on agriculture in this area occurred after the discovery of *in vitro* genetic engineering and the use of transgenic plants. Biotechnology as a new era, 20 years into its development, is showing its positive impacts in production agriculture and new food crops.

It could be said that we still have some distance to travel before a larger contextual effect can be felt. In general there is confidence that judicious and timely applications of modern genetics to plant science will be an important driver of world agriculture. Given the rising number of people it is also understood that abundance of transgenic food plants does not

Table 1 Food production through fungal biotechnology

Amino acids	Food pigments
Beverages	Food enzymes
Dairy products	Mushrooms
Digestive aids	Organic acids
Dough	Single-cell protein
Ethnic foods (kefir, koji, miso, tempeh, etc.)	Vitamins

necessarily translate into abundant supply for people. To feed the world population, we must strive to overcome global deficiencies in food transportation, and storage in many countries as well as its affordability (Khachatourians 2002). Certainly, agricultural biotechnology can provide part of the answer, but global sociopolitical factors, including ethics of farming, farmers, corporate agribusiness, world trade organizations and states, and international treaties and enforcement agencies, will also be influential.

3 THE BALANCING ROLE OF FOOD TECHNOLOGY

Historically applications of agronomic practices have been the most significant exploitation for food production. Many countries rely on importation of food grains, fruits, and vegetables. However, many nations have shortfalls of food crops that are exasperated by unpredictable global climate change. The world production of grains per person has remained at about 300–340 kg since 1970s. Of the three—wheat, rice, and corn—the world wheat carryover stock was at 78 days in 1999. The food security threshold is 70 days. The supply of rice stocks was down to 42 days of consumption; consumption had been on the rise for 26 consecutive years since 1973 (Khachatourians 2002). A decade has passed since the United Nations' Framework Convention on Climate Change, and even with three major gatherings, Rio Earth Summit, Kyoto Protocol, and this year's Johannesburg World Summit on Sustainable Development, we are no where near adoption of a policy that would help sustain world development trends, whether food and agriculture, or food security and safety, or population increase.

The summer of 2002 brought drought to parts of the North America and flooding to parts of Europe and Asia which seriously affected world wheat and other grains production, subsequent grain harvesting, and overall food production markets. If we add the role of water in irrigation, sustainability of food and agriculture trends for production, and its scarcity, the outlook deteriorates dramatically. A total of 1000 ton of water is required to produce 1 ton of grain. Aquifer depletion or contamination is an ignored but real threat to plant based food production. Various contributions of biotechnology and construction of transgenic plants and applications of microbial biotechnology are one of the known

trends for building production capacity in the next 25 years (Khachatourians 2002; Khachatourians et al. 2002).

It is becoming increasingly clear that the once known questions and hence answers of food production are no longer that well known or understood (Khachatourians 2001). Other unknown elements of further increase in appropriate food production are the understanding of the shift that has occurred in the paradigmatic aspects of food science, from the delicate balance of the interplay in organismic biology, Availability of germplasms, and Applications of biotechnology to co-operation of environment and issues of food governance, science, safety, and economics (Phillips and Wolfe 2001). While plant and animal biomass based food production is advancing more rapidly than in past decades, new understandings of the processes involved in production and post production events, especially by biotechnological means can and add value to post production agriculture to foods and new products by microorganisms. Innovation, invention, and investments in biotechnology will continue to impact the food industry and possibly help to maintain equilibrium of food production and consumption (Phillips and Khachatourians 2001).

4 FUNGAL BIOTECHNOLOGY IN FOOD PRODUCTION

No matter how anecdotal the evidence, even the ancient societies recognized the use of fungal technology, in relationship with their agriculture and food. Knowledge of fungal diversity and distinguishing beneficial fungi for the biotransformation of food ingredients, helped to sustain and extend our food source. In spite of the powerful toxic secondary metabolites of many fungi, humanity survived these fungi and through innovative use of the beneficial micro and macro fungi found particular culinary and other uses of the mushrooms (see: this volume, chapter by Rai).

The importance of the fungi relates to their particular modes of growth and proliferation, production, and secretion of extracellular enzymes, peptides of novel functions (taste, physiological functions, and antimicrobial activity) and secondary metabolites and antibiotics consequential to prevention of infectious microbial growth. The molecular biologists of the day work with a few well-studied fungi, (Table 2) but many others from the 72,000 species wait in line to be studied (Hawksworth 1991).

Through advances in the fungal biotechnology we understand fungal role in the new age of applied molecular biology. As we have a growing base of some 10,000 fungal species in various collections and the public domain literature we have some of the relevant knowledge to cross connect to biotechnology for exploitation of fungi for food and ingredients manufactured for human benefit. The knowledge base in mycology is in a good position to lead the new biotechnology of food.

Table 2 Representative fungal species in food biotechnology

Genus species/synonym	Genus species/synonym
Yeasts	Filamentous fungi
<i>Candida</i>	<i>Aspergillus</i>
<i>Maltosa</i>	<i>nidulans</i>
<i>Antarctica</i>	<i>niger</i>
<i>Pseudotropicalis</i>	<i>oryzae</i>
<i>Shehatae</i>	Mucor
<i>utilis</i>	<i>hiemalis</i>
<i>Debaryomyces hansenii</i>	<i>miehei</i>
<i>Kluyveromyces</i>	<i>pusillus</i>
<i>lactis</i>	<i>Penicillium</i>
<i>marxianus</i> var. <i>marxianus</i>	<i>album</i>
<i>Pachysolen tannophilus</i>	<i>camemberti</i>
<i>Phaffia rhodozyme</i>	<i>caseicolum</i>
<i>Pichia stipitis</i>	<i>roquefortii</i>
<i>Saccharomyces</i>	<i>Rhizopus</i>
<i>bayanus</i>	<i>arrhizus</i>
<i>carlsbergensis</i>	<i>cohnii</i>
<i>cerevisiae</i>	<i>delemar</i>
<i>exiguus</i>	<i>niveus</i>
<i>lipolytica</i>	<i>oligosporus</i>
<i>rosei</i>	<i>oryzae</i>
<i>rouxii</i>	Edible mushrooms
<i>sake</i>	<i>Agaricus bisporus</i>
<i>uvarum</i>	<i>Agaricus campus</i>
<i>Yarrowia lipolytica</i>	<i>Lentinus edodes</i>
	<i>Pholiota nameke</i>
	<i>Pleurotus ostreatus</i>
	<i>Volvariella volvacea</i>

Changes in the functional features of the starting materials leading to food products and processes are the other side to the fungal biotechnology. It is expected that efforts in the public and private sector research establishments should provide new inputs for food production. On the output side, it is expected that increased productivity, higher value added, improved quality and shelf life, and spoilage protection will impact market situation. These products and processes for food crops and agricultural practice are not only challenging for the scientific community interested in mycology but also for public health and commerce. From various estimates, the values of sales of mycology-based products can run into tens of billion of dollars, projected by this decade, certainly not an insignificant figure.

5 FOOD BIOTECHNOLOGY: PRODUCTS AND PROCESSES

Whether through traditional or present day biotechnological routes, fundamental applications of fungi have made a substantial impact on our foods (Hui and Khachatourians

1995; Khachatourians and Arora 2001; 2002). Historically, applications of mycology in agriculture and food have been exploited by many countries and ethnic groups and are now being enhanced by several tools and concepts of biotechnology (Hui and Khachatourians 1995; Pointing and Hyde 2001; Singh and Aneja 1999). In recent years much are emerging in the developed countries that serves as new learning opportunities in application of fungal biotechnology for the food and environment. As it can be seen from chapters of this book, there are more rapid advances and new understandings of the processes and new products aided or deterred by fungal biotechnology. In general, all areas of food technology, whether pre- or postharvest food crop management, transformation and value differentiation of commodities, increased production efficiency, increased value-in-use of animal and plant food and nonfood markets will be affected by fungal biotechnology.

As evident from the first section of this compendium, a large proportion of the innovations in food biotechnology come from the renewed assessment of fungal physiology, biochemistry and genetics in order to determine methods and options for manipulations at the molecular level. Outside the natural sciences, agricultural biotechnology of today also requires the convergence of several disciplines from production strategies, process engineering, commerce, and international law. Indeed it is the entire process of science, investment, inventions, and innovations that are the interdisciplinary and transdisciplinary characteristics of the new biotechnology (Phillips and Khachatourians 2001).

Advances in ingredient subdisciplines of mycology as in the past will remain the drivers of applied agricultural research. With new interests there will be major investment focused on generating discoveries and their applications towards both conventional and biotechnology oriented useful products and processes or services.

In the sections to follow we will present certain highlights of basic and applied mycology that are exciting developments and which impact the cross connection with biotechnology for applications to agri-food (Rajak 2000). Needless to say specific developments in fields ancillary to mycology should continue to be of significant impact on the new applied mycology and biotechnology products and processes.

5.1 Food-Use Enzyme Production

Specific enzymes create particular functional and hence value contributions in foods. The predominant source for global market demand of enzymes comes from fungi and bacteria. From a multitude of enzymes synthesized and many secreted extra-cellular, a few occupy the dominant role in food ingredient production (MacCabe et al. 2002). Biotechnology continues to enhance the yield and functional attributes of fungal enzymes (see: this volume, chapters by Saxsena and Malhotra, and Viniegra-Gonzalez). The most important aspect of this realization has been in the application in the context of food production/processing including

immobilization for catalysis and secondarily as a source for commercial “digestives” containing enzyme(s) for consumption. Yoshimaru et al. (2000) describes improvement of the digestion in pigs by using microencapsulated aspartase from *Aspergillus usamii* and *A. shiro-usamii*. The production of these enzymes occurs by batch liquid or solid/semisolid fermentations. The choice is often determined by considerations for maximum quantity, activity, purity, or for cost and efficiency.

5.2 Organic Ingredients Production

Organic acids, vitamins, nonvolatile and volatile flavor organic molecules (e.g., vanillin and 2-heptanone) are important compounds in food production and technology. They serve as food ingredients or as precursors for food ingredients. The major organic acids produced and used in the largest volumes function primarily as food acidulants. The means for organic acid and production methods rely on bacteria and fungi. If these ingredients are used as a food ingredient, they must have a GRAS (generally regarded as safe) status. Although the use of *Penicillium* was the primary source for many organic acids, in recent years *Aspergillus niger* has become the preferred organism and riboflavin oversynthesizing strains of *Pichia guilliermondii* the preferred source.

5.3 Fermented Foods and Beverages

Industrial yeasts are involved in the production of many foods and drinks. The edible products, cheese and bread, and the potable alcohol products, beer, wine, and spirits, depend on yeast-based fermentation. The techniques of biotechnology developed in recent years enable the engineering of industrial yeasts including the multiploid yeasts, which are extremely difficult to manipulate by traditional genetics. Normal yeasts are unable to completely convert starch to alcohol because of their inability to degrade the starch beyond its branch points. Starch debranching-enzymes and their genes have been identified and engineered into the industrial yeast. As a result, it is possible to completely convert starch to alcohol, resulting in the production in a single natural step, light beer. Other genes could be incorporated into industrial yeasts to improve the efficiencies of manufacture of cheese, bread, wine, and spirits and to make other useful products (Hui and Khachatourians 1995). New technology development in fermentation can be powerful in contributing to agri-food industries. Kourkoutas et al. (2002) described a high temperature alcoholic fermentation of whey using *Kluyveromyces marxianus* cells immobilized on delignified cellulosic material under batch conditions. They found that volatile byproducts and higher alcohols in very low levels led to a product of improved characteristic aroma. The potential use of various agri-food enzymes in processing raw material for

the production of novel foods and drink represents an untapped resource for fungal biotechnology.

In certain food production schemes, food additives and ingredients obtained through fungal fermentation technology contribute much to aroma and flavors. Fungi grown in either liquid cultures or in solid-state fermentation have the advantages of *in situ* contribution to the value of foods (see Arora 2004, chapters by Saxsena and Malhotra; Nigam, Robinson, and Singh; and Vinigera-Gonzalez). Several specific aspects of fungal biotechnology's contribution to functional attributes including taste and smell of foods are discussed in depth in the following chapters (see: this volume, chapters Castrillo and Ugalde; Agrawal; and Hansen and Jakobsens; see also chapter by Avalos, Arora 2004).

5.4 Fermentation Technology and Downstream Processing

Yeasts and filamentous fungi were traditionally employed in the production of alcoholic beverages and fermented foods over centuries (Hui and Khachatourians 1995; Rajak 2000). Yeasts (mainly *Saccharomyces*) have been used worldwide for brewing and baking for thousands of years. Likewise, filamentous fungi have been traditionally used for preparing mold-ripened cheeses (mainly *Penicillium* spp.) in Europe and soybean-based fermented foods (mainly *Aspergillus* spp.) in the Orient. On the other hand, edible mushrooms (such as *Agaricus*) have been used worldwide for direct consumption since times immemorial (Hudler 1998; Pointing and Hyde 2001; Rajak 2000; Singh and Aneja 1999). With passing time, these fermentation techniques were scaled up and made more efficient with respect to engineering theories and practices. Main outcomes of the evolution of food processing and production activities, have been the introduction of interdisciplinary natural and engineering concepts, for example, better equipment design, heat and mass transfer systems, feedstock supplementation system, product recovery, effluent and waste management (Thassitou and Arvanitoyannis 2001, for an extended coverage see: this volume, chapter by Schliephak et al.), computerization and automation, and finally hazard analysis critical control points (HACCP) and quality assurance. Ancillary to the above developments has been the area of food packaging storage, transportation, and distribution system design. Overall, there has been incredible integration of food science, microbiological, engineering, and industrial R&D activities.

Fungi, because of their unique mycelial structures have challenged bioengineers to reinvent fermenters and downstream processing instruments. Basic research aimed at better fungal uses for food production have synergistically aided many other aspects of fungal biotechnology from life sciences molecules to a variety of polymer sciences. More recently advances in natural science and engineering have led to the application of biosensors; computer control, logistics, and real

time data collection and analysis; on line analytical instrumentation; and the use of new materials for processing.

6 FOODBORNE FUNGAL PATHOGENS AND MYCOTOXINS

It is estimated that some 400 fungi and many of the phytopathogenic ones reduce or threaten the availability and safety of food. From the above, at least 20 are confirmed producers of food related mycotoxins and antinutrients (see: this volume, Chapter 6). Many vegetables, fruits, and seeds lose their nutritive and other qualitative values due to loss of moisture, infection with spoilage microorganisms, and senescence. These types of wastage can occur during transport, handling, and redistribution. Food crops and their products can be infected with single or multiple species of toxigenic fungi. As a result food commodities can be contaminated inadvertently with levels of toxic metabolite(s) often ranging in PPB to PPM levels (Jones et al. 1995; Koshinsky and Khachatourians 1992; Koshinsky and Khachatourians 1994).

Exposure to these metabolites whether singly or in combination results in toxopathological manifestations and even death. Even at subthreshold levels, multiple mycotoxins, by their interactions with multiple sites and targets often produce devastating synergistic effects on living cells and whole animals (Koshinsky and Khachatourians 1992; Koshinsky and Khachatourians 1994; Koshinsky et al. 2000). The historically significant and still prevailing food consumption related threat to public health comes from the fungal alkaloids that lead to ergotism. The fungus, *Claviceps purpurea* is prevalent in the cool climates where rye is grown. *Claviceps africana* has been found to spread through sorghum in North and South America, Australia, and Africa (Bandyopadhyay et al. 1998). Sorghum is the fifth most important cereal crop in the world.

Huge challenges are posed by mycotoxins such as aflatoxins, trichothecenes, and fumonisins that can be addressed by fungal biotechnology. Identification of mycotoxin biosynthesis and their genes, mycotoxin catabolism and biotransformation and their genes could have tremendous value. So far, however, breeding of corn and cereal grain plants through conventional genetics for resistance have been attempted but remains unsuccessful (Medianer 1997). This is possibly due to multiple modes of action of some of these toxins and hence polygenic nature of resistance (Khachatourians 1990; Koshinsky et al. 1998; Woytowich et al. 1997). Perhaps with the isolation of target specific genes a better and fuller resistance could be achieved.

Saprophytic and pathogenic fungi are a major detriment to freshness of fruits and vegetables and safety. Application of antifungal peptides and antimicrobial peptides (Nigam et al. chapter in Arora 2004; Woytowich and Khachatourians 2001; see: this volume, chapter by Ray and Liewen) could significantly change our options in management of spoilage

fungi. A new avenue for applied research is the application of genomics, proteomics, and bioinformatics towards intervention with fungal developmental genes for enhanced functionality, for controlled ingredient delivery, or spoilage of foods. As the knowledge of the regulation of fungal gene expression advances, it is expected that strains will be designed for expression of commodities of high impact in world trade.

Recent advances in diagnostic biotechnology have revolutionized the procedures used in the identification of food fungi. Biochemical identification assays have been miniaturized and through automation and uses of robotics have become faster, reliable, and cost affordable. Rapid identification of fungi and yeasts from foods has become less cumbersome because of ease in sequestering of target fungi from the food ingredients and interfering compounds. In addition, biochemical tests which traditionally have been used in the identification of yeasts and filamentous fungi have been greatly aided by the introduction of polymerase chain reaction (PCR) technology.

Research and development work on understanding of fungal presence in food products and processes are advancing rapidly. For the most part, the goal of research in this area is to decrease process costs. However, fungal biotechnology will enable improvement in health and safety related issues of foods such as better shelf life, decreased threats to human health, and increased perceived healthfulness of the food.

7 CONCLUSIONS

Clearly as we move through the 21st century, the prospects for food production, storage, transportation, and processing will change by the same forces that are impacting on all other facets of economy. The benefits of R&D in fungal biotechnology for enhanced food production; security and safety will benefit everyone. The support for fostering a research environment conducive to the above goal must therefore be responsibilities for everyone whether in the public or private sector. Fungal biotechnology can ensure harnessing of the potential of fungi while ensuring our foods are free from fungal pathogens, mycotoxins, mold allergens, and other problems of quality loss during storage. Fungal biotechnology has the potential of being a primary driver of world food production and its quality.

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Fungi and Fermented Food

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1 INTRODUCTION

Fermentation is a process in which complex compounds including carbohydrates, proteins, and fats are broken down to simpler forms under aerobic or anaerobic conditions and have been used by the food industry for centuries. The products of fermentation can be used as a food source itself or as an additive. Fermentation has many roles including detoxification, adding nutritional value, creating aromas or flavors, reducing cooking time and so fuel consumption, and some fermented foods may even have therapeutic value. A knowledge of microbiology, genetics, and biochemistry is important in the food fermentation industry as yeasts, molds, and bacteria play an integral role in food fermentation. Improvement of these microorganisms by genetic manipulation may result in higher-quality fermented food and a considerable amount of work has been done in the screening of useful microorganisms. Further development of fermentation equipment, toxin measurement, prevention of food spoilage, nutritional analysis, flavor and taste assessment, and addition of colors and additives are underway to attain the maximum potential from this process. Many of the issues are covered in a book edited by Steinkraus (1996) and in another two edited by Joshi and Pandey (1999).

Although some fungi are pathogenic to plants and animals and thus bring about economic losses, many of them are beneficial to mankind especially in food production. In this chapter, different types of fermented food with fungal involvement in the fermentation process are described. It is worth noting that different countries may have some similar indigenous fermented food as well as varieties that are unique to that country.

2 ACID-FERMENTED LEAVENED BREAD AND PANCAKES

These are highly accepted as food. The acid fermentation makes the food resistant to spoilage and development of food toxins and less likely to transfer pathogens.

(a) *Indian Idli*: Idli is the Indian equivalent of the Western sourdough bread. It differs from sourdough bread as first leavening is achieved by bacterial instead of yeast activity and secondly wheat or rye is not used as source of protein to retain carbon dioxide during leavening. Idli is prepared by bacterial fermentation of batters produced from washed, soaked rice, and dehulled black gram. After fermentation overnight at around 30°C, the batter is poured into cups of an idli steamer, placed in a covered pan and steamed for about 15 min until the idli cakes are soft and spongy. The texture and flavor can be achieved by replacing rice by other cereal grains, and a variety of legumes can be used instead of black gram. The lactic acid bacterium *Leuconostoc mesenteroides* is important for batter leavening. Yeasts are not essential, although when added they will contribute to leavening, flavor, and increasing levels of the vitamins thiamine and riboflavin. The yeasts used in fermentation for production of idli include *Saccharomyces cerevisiae*, *Debaromyces hansenii*, *Hansenula anomala*, *Trichosporon beigelii*, *Oidium lactis*, *Torulopsis holmii*, *Torulopsis candida*, and *Trichosporon pullulans* (Soni and Sandhu 1999); (b) *Philippine Puto*: Puto is similar to Indian Idli except that there are no legumes in Puto, so the flavoring is excluded. It is produced by grinding soaked rice grains in water, adding sugar and a starter culture containing *L. mesenteroides*, *Streptococcus faecalis*, and *S. cerevisiae*, so allowing the mixture to ferment. This fermentation lasts for about 15 h, before adding sodium hydroxide (lye) and more

sugar. A second fermentation continues for about 5 h before steaming for 30 min. *S. cerevisiae* is a minor component until the final stage of the fermentation in which it can reach as much as 18% of the total population, resulting in a small amount of ethanol. The yeast together with *L. mesenteroides* may play an important role in leavening the batter (Rosario 1987); (c) *Sri Lankan Hopper*: Rice and wheat flour, either alone or mixed, are added to sugar, coconut water, coconut toddy, and inoculum (a collection of yeast and acid-producing bacteria) or baker's yeast (*S. cerevisiae*). After the mixture has formed into a stiff batter, it is covered with a piece of wet cloth and allowed to stand at room temperature for about 12 h to allow carbon dioxide to be produced by the yeast. Following this fermentation, coconut milk and salt are added. Sodium bicarbonate may also be introduced to raise the batter during cooking. The mixture is then poured into an oiled hot pan and baked for about 4 min. A longer fermentation period produces a better flavor. Nutritionally, the yeast provides proteins and B vitamins, and the coconut supplies some proteins, long-chain fatty acids, and flavor (Ekmon and Nagodawthana 1977); (d) *Ethiopian Enjera*: Enjera, also known as injera, wanjera, manjeriya, and kissra habashiya, is made from teff (grain of a type of grass related to love-grass), wheat, barley, sorghum, corn or a mixture of them. It is a fermented sour leavened pancake-like bread. The teff flour is mixed with water and starter culture before being incubated for 1–3 days to produce a paste. The fermented paste is then mixed with water, boiled, and cooled down before baking for several minutes to give injera. A number of fungi including *Aspergillus*, *Candida*, *Hormodendrum*, *Penicillium*, *Pullaria*, *Rhodotorula*, and *Torulopsis* species have been identified as being used in this process. It has been suggested that *Candida guilliermondii* is the primary fermenting organism although evidence has been presented that gram-negative rod-shaped bacteria may be responsible for the initial fermentation (Stewart and Getachew 1962); (e) *Ghanian Kenkey*: Kenkey appears in the form of sour maize dough balls or cylinders enclosed by maize husks or plantain leaves. To prepare kenkey, maize kernels are washed and soaked in water before being ground into fine particles. The powder is moistened with water, packed, and covered tightly to ensure anaerobic conditions before leaving to ferment for 1–3 days. A portion of the dough is then precooked, while salt is added to the rest of the mixture. The precooked dough is mixed with the remaining dough, made into balls and wrapped with maize husks or plantain leaves prior to thorough cooking. This fermentation process is uncontrolled and initially involves *Aspergillus*, *Penicillium*, and *Rhizopus* species. *Saccharomyces* sp. have also been detected and as yeasts esterify organic acids and alcohols, they give a distinctive aroma to kenkey. Bacteria are also involved in this process. Gram-negative, catalase-positive bacteria appear in the beginning but soon disappear, to be replaced by gram-positive, catalase-negative bacteria. This microbial activity contributes to its nutritive value by increasing the content of thiamine, riboflavin, and protein in kenkey (Christian 1970); (f) *Nigerian Gari*: Gari is a fermented cassava product. Peeled

cassava roots are washed, grated, and packed into a bag that is weighted to squeeze the juice out. Natural fermentation is allowed to proceed for several days resulting in a lower cyanide content and softer mushy extreme of the cassava. The yeasts including *Candida* sp. together with bacteria (*L. mesenteroides*) are responsible for the flavor and changes in acidity during fermentation (Oneyekwere et al. 1989); (vii) *Nigerian Kamu*: Kamu is a starch-cake food produced from fermented millet. The yeasts, *S. cerevisiae* and *Candida krusei* and the lactic acid bacteria are used in fermentation. The mixture of soaked millet grains, pepper, ginger, and fruit is wetmilled with water and sieved. Kamu is the sediment obtained after the filtered liquor is allowed to settle for several hours and can be used to make porridge (Oyeyiola 1991); (h) *Nigerian Ogi*: Ogi is made by fermenting cereals including maize, sorghum, and millet that not only give them different flavors, but also result in different colors. Fungi and bacteria are included in the fermentation. *Aspergillus*, *Cephalosporium*, *Cerecospira*, *Fusarium*, *Oospora*, *Penicillium*, *Rhizopus*, *Rhodotorula*, and *Saccharomyces* are fungi associated with fermenting maize. The yeasts contribute to the flavor of Ogi. The bacteria involved are *Lactobacillus plantarum*, *Lactobacillus brevis*, *Acetobacter* sp., *Corynebacterium* sp., and *Lactobacillus plantarium*, which is the predominant organism producing lactic acid (Akinrele 1970); (i) *Sudanese Kisra*: Kisra is bread or stiff porridge prepared by fermenting sorghum flour. To prepare Kisra, sorghum grains are subjected to dry milling and sieving before fortification with wheat or millet grains. The mixture is then milled, and mixed with water before a starter culture is added to start fermentation. After half a day at around 37°C, water is then added before baking. Many microbes are involved in this process, Yeasts, *Candida intermedia*, *D. hansenii*, and *S. cerevisiae* multiply throughout the fermentation. The molds of the fungal genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Rhizopus* are also found. The bacteria involved include *Erwinia ananas*, *Klebsiella pneumonia*, and *Acetobacter* sp. Dough leavening is caused by *Acetobacter* converting alcohol to acetic acid and yeasts produce CO₂ (Mohammed et al. 1991).

3 FERMENTED MILK PRODUCTS

The advantages include easy production, improved keeping quality, better digestibility and nutritive value, and therapeutic potential. Only the acid-tolerant bacteria can grow during milk fermentation.

(a) *Egyptian Kishk* is a fermented milk-wheat mixture that is stored as dried balls. To prepare kishk, wheat grains are softened by boiling, air-dried, and then ground. Milk is added to the ground wheat to produce a paste that is rolled into balls. Spices may be added to the balls before they are dried in the sun. The numbers of molds, yeasts, and bacteria have been examined. *Penicillium* and *Mucor* molds are found up to 10³ g⁻¹. Yeasts are found in 10⁴–10⁶ g⁻¹ concentration but bacteria make up the highest numbers, which also contribute

to the aroma with *Lactobacilli* found in 10^8 g^{-1} (Mahmoud 1977); (b) *Russian Kefir* is an acidic, mildly alcoholic milk made from cow, goat, or sheep milk that is able to keep longer. The predominant yeasts involved in fermentation include *T. holmii* and *S. delbrueckii* although *Candida kefir* and *S. cerevisiae* are the yeasts that are commonly isolated (Marshall 1984). During the preparation of kefir, Kefir grains are added to pasteurized milk and incubated at 18–25°C for 1–2 days. The mixture contains Kefir grains, which consist of yeasts and lactic acid bacteria held together with the help of a polysaccharide gum and these are removed by sieving; (c) *Russian Koumiss*: This is produced from ass milk, camel milk, or mare milk. Lactic acid bacteria and the lactose fermenting yeasts *Torula kumiss* and *Saccharomyces lactis* cause fermentation (Kosikowski 1982). Fresh milk is first heated at about 90°C for 5 min and cooled before inoculation of starter and incubation at 28°C. The incubate is agitated every 24 h and then cooled to form koumiss. Koumiss has both nutritive and therapeutic values and has been reported to be effective in the treatment of pulmonary tuberculosis in Russia; (d) *Lebanese Labneh*: This is formed from goat or sheep milk. Lactose-fermenting yeasts and lactic acid bacteria cause fermentation; (e) *Cheese*: Cheese-making comprises several steps: preparation of milk; addition of starter and ripening (souring), renneting and coagulation, and ripening/curing of cheese curd into the final product. Molds such as *Penicillium roqueforti*, *P. glaucum*, *P. camemberti*, *Geotrichum candidum*, and *Mucor racemosus* are used in ripening. Different combinations of molds and bacteria are used for different varieties of cheese; (f) *Indian Dahi*: Dahi is produced by lactic fermentation of milk as a bioproduct of alcoholic fermentation by yeast. Raw milk is boiled for a period of a few seconds up to 10 min, then cooled before addition of starter and incubation at room temperature for 8–16 h (Baisya and Bose 1975).

4 ALCOHOLIC FOOD AND BEVERAGES (MOSTLY CEREAL-BASED)

These are primitive beers and wines prepared by villages in the developing countries. They are cloudy slurries containing residues and microorganisms including yeasts, and hence are a source of B vitamins, proteins, amino acids, and calories.

(a) *Ethiopian Tej*: This is a home-processed honey wine fermented by *Saccharomyces* species present in the environment that convert sugars to ethanol. To make Ethiopian tej, honey, water, and hop stems are fermented in a pot for about 1 week at high ambient temperature or 2 weeks at low temperature with stirring daily. The mixture is then filtered through cloth several times and the final filtrate is collected (Vogel and Gobeze 1977); (b) *Kenyan Urwaga*: This is a slightly sour drink prepared from bananas, maize, millet, or sorghum. Yeasts and lactic acid bacteria are involved in the process of fermentation. To make Urwaga, green bananas are ripened in a covered pit lined with banana

leaves. The peeled bananas are mixed with grass and the juice is forced out by squeezing by hand. A roasted flour mixture made from maize, sorghum and millet is mixed with the banana juice and fermented in the covered pit for half a day or one (Harkishor 1977); (c) *African Kaffir (Kaffircorn) (Sorghum) Beer*: This has a sour, yogurt-like flavor. The brewing process involves lactic acid fermentation and an alcoholic fermentation. Lactic acid fermentation by lactic acid bacteria causes souring. The yeast *S. cerevisiae* is used for alcoholic fermentation (Hesseltine 1979); (d) *Nigerian Pito*: This is a slightly bitter, sweet-sour beverage with a fruity flavor produced by fermentation of maize or sorghum. The molds *Rhizopus oryzae*, *Aspergillus flavus*, *Penicillium funiculosum*, and *Penicillium citrinum* play an undefined role in Pito fermentation. The bacteria *Leuconotoc* sp. and *Lactobacillus* sp. and the yeasts *Saccharomyces* and *Candida* spp. are also present during fermentation. Amylases from the germinated maize or sorghum grains and the molds present on the grains cause hydrolysis of starch in the grains to form maltose and glucose. The yeasts ferment the sugars to form ethanol. The lactic acid bacteria produce lactic acid (Ekundayo 1977); (e) *South America fermented maize chichi*: This has a ciderlike flavor. Molds including *Aspergillus* and *Penicillium* spp., yeasts including *S. cerevisiae*, *S. apiculata*, *S. pastorianus*, and *Mycoderma vini*, and lactic acid bacteria are present. Amylase needed for hydrolysis of starch is produced from germinated maize or from human saliva. Chicha produced using saliva reportedly has a better flavor (Gomez 1949); (f) *Mexican Tesguino*: This is prepared by fermentation of germinated maize or maize stalk juice. To make Mexican Tesguino, germinated maize kernels or maize cane stems are first mixed with water and boiled. Catalysts, which include yeasts, vitamins, enzymes, or growth factors, are added before fermentation. *S. cerevisiae*, *C. guilliermondii*, and *H. anomala* are important in the alcoholic fermentation of Tesquino (Lappe and Ulloa 1989); (g) *Philippine Basi*: This is sugar cane wine. Sugar cane juice is boiled and fermented with a mixture of molds, yeasts, and bacteria in an earthenware jar for 6 months to 1 year (Sakai and Caldo 1984); (h) *Philippine Tapuy*: This is a sweet rice wine. To make Philippine Tapuy, the rice is washed, cooked, cooled, and placed in a clay pot. Powdered bubod is then inoculated for fermentation which lasts for 2–3 days. The rice and the liquid collected on top can be consumed separately or together. The amylase-producing yeasts *Saccharomyces uvarum* and *Endomycopsis (Saccharomycopsis)* and the lactic acid bacteria are the dominant organisms present (Sakai and Caldo 1983); (i) *Japanese Sake*: This is a rice wine made from steamed rice overgrown with *Aspergillus oryzae* mycelium. The yeast *Saccharomyces sake* also plays a role. The process of sake brewing first involves the preparation of polished, steeped, and steamed rice, followed by the preparation of starter consisting of yeast and lactic acid bacteria. Consecutive addition of more steamed rice, rice koji (*A. oryzae*) and water are then followed by the main fermentation, which takes place in an open system without the exclusion of nonstarter microorganisms. The combination of

hydrolysis of starch by *A. oryzae* and slow fermentation by the yeast *Saccharomyces sake* at a temperature below 10°C is referred to as parallel fermentation, which gives rise to the high (15–20%) ethanol content of sake (Murakami 1972); (j) *Tea Fungus/Kombucha*: Several types of yeast including *C. guilliermondii*, *Pichia membranefaciens*, *Saccharomyces* sp., and *Torulopsis formata* are present in Japanese tea fungus. *Candida obtuse* and *Kloeckera apiculata* are present in Formosan tea fungus (Kozaki et al. 1972). Reiss (1994) reported that tea fungus consists of *Acetobacter xylinum* and the following yeasts in symbiosis: *Pichia* sp., *Saccharomyces ludwigii*, and *Saccharomyces pombe*. The yeasts produce ethanol from the sugars added and *Acetobacter* oxidizes ethanol to acetic acid. It is believed that tea fungus enhances hepatic detoxification and inhibits tumorigenesis; (k) *Chinese Spirits, Wines, and Beers*: Some are made from barley or wheat and brans overgrown with *Aspergillus* while others are made from rice or rice bean overgrown with *Mucor* or *Rhizopus* (Chen and Ho 1989); (l) *Chinese Lao-Chao*: This is made from fermentation of glutinous rice. The fungi and yeasts grown on rice flour include *R. oryzae*, *Rhizopus chinensis*, and *Amylomyces rauxii* (Wang and Hesseltine 1970). The yeasts ferment the starch; (m) *Indian Ruhi*: This is prepared by fermentation of boiled rice. Boiled rice is spread, cooled, and mixed with the inoculum, which is comprised of molds belonging to genera *Rhizopus* and *Mucor* and yeasts (Dahiya and Prabhu 1977). It is then poured into a basket. The rice liquefies upon fermentation and the liquid is collected in a pot beneath the basket. (n) *Indian Madhu*: This is made by fermentation of boiled rice by lactic acid bacterial spp., *Mucor* and *Rhizopus* spp. (Dahiya and Prabhu 1977). Sugars formed by the hydrolysis of starch are fermented to form alcohol and lactic acid; (o) *Whisky*: *S. cerevisiae* is involved. Corn, rye, and barley are used. There are several types (a) Scotch whisky produced from water and malted barley to which only whole grains of other cereals may be added. (b) Irish whisky made from unmalted barley. Compared with Scotch whisky it has higher ethanol content and a stronger flavor but lacks the peat characteristics. (c) Canadian whisky with a light flavor and made from corn, rye, and barley malt. The spirits must be aged for a minimum of three years. (d) American rye whisky containing at least 51% rye, American corn whisky containing at least 80% corn, American light whisky containing a large percentage of corn, and Bourbon whisky containing at least 51% corn (Russell and Stewart 1999).

5 FRUIT-BASED ALCOHOLIC BEVERAGES

A variety of alcoholic beverages is made from fruits. The quality of fruit wine depends on the fruit variety, maturity of fruit, yeast strains, other vinification practices, and the method of preservation.

(a) *Mango Wine*: Mangoes are first pulped, Pectinase is then added followed by fermentation with *S. cerevisiae* var. *ellipsoideus* (Joshi et al. 1999); (b) *Jambal wine*: Crushed

Jambal fruits are diluted and the must ameliorated with cane sugar. Diammonium hydrogen phosphate, sulfur dioxide, and pectinol enzyme are introduced, then follows fermentation with the yeast *S. cerevisiae* (Joshi et al. 1999); (c) *Coconut toddy*: This is produced by naturally fermenting the inflorescence sap of coconut palm in open pots for 2 days. *Candida* spp., *Kloeckera javanice*, *Saccharomyces chevelier*, *S. exiguus*, *S. marxianus*, *Schizosaccharomyces pombe*, and *Pichia ohmeri* are involved in the fermentation process; (d) *Palm wine*: *S. cerevisiae* is the yeast usually instrumental in fermentation. Other fungi including *Schizosaccharomyces pombe*, *Candida*, *Mycoderma*, *Aspergillus*, *Mucor*, *Pichia* sp., and *Rhizopus* sp. are detected together with lactic acid bacteria in Nigerian wine (Faparusi 1977). Atputharajah et al. (1986) identified *Candida*, *Pichia* and *Saccharomyces* species as the major yeasts responsible for the natural fermentation of coconut palm sap. The wine is a milky suspension of live bacteria and yeasts. It has a sweet taste and exhibits vigorous effervescence due to fermentation. The wine is made from sap collected from a cut on middle-aged flowering palm trees. It is collected in earthenware pots containing the bacteria and yeasts and any left-over toddy; (e) *Indian jackfruit wine*: To make jackfruit wine, seeds are removed from peeled ripe jackfruits and the pulp soaked and ground in a bamboo basket. The extract is collected in earthenware pots. A small amount of fermented juice is added as inoculum and the extract is allowed to ferment at 18–30°C for a week. The yeast involved in fermentation appears to be *Endomycopsis* (Dahiya and Prabhu 1977); (f) *Date wines*: Different types of date wine involve different fungi that include *Torulopsis*, *Saccharomyces*, and *Candida* spp. and also different bacterial species including *Acetobacter*, *Bacillus*, *Gluconobacter*, *Klebsiella*, and *Leuconostoc*. Dates are either soaked in lukewarm water and allowed to ferment for about 4 days, or boiled to form a syrup which is then allowed to ferment for about 3 days in a cloth bag of either sorghum malt or a mixture of ginger and cinnamon immersed in the syrup (Ali and Dirar 1984); (g) *Plum wine*: To prepare plum wine, water and starter culture are added to the plums and fermented for about 10 days before pressing for juice. The yeast *S. pombe* is used for deacidification of the acidic plum pulp. *S. cerevisiae* is used for alcohol production (Joshi et al. 1999); (h) *Mead (honey wine)*: *S. cerevisiae* is used for alcoholic fermentation in the production of mead and wine from apples, pears and plums where honey is utilized as a source of sugar (Joshi et al. 1999); (i) *Kiwi fruit wine*: Kiwi fruit juice is clarified with the help of pectolytic enzyme with the resulting generation of an intense fruity aroma. The juice is highly acidic and has low sugar content, making it necessary to ameliorate the juice. *S. cerevisiae* is added to the must before fermentation. The ascorbic acid content is preserved by SO₂ at low concentration (Joshi et al. 1999); (j) *Apricot wine, litchi wine, sparkling plum wine*: *S. cerevisiae* is involved. (a) Apricot wine: made by diluting apricot pulp with water (1:2 by volume), addition of 0.5% pectinol and 0.1% diammonium hydrogen phosphate and fermentation with *S. cerevisiae*, (b) litchi wine: peeled litchi fruits are dipped in sugar solution for

4 h at 50°C. The fruits are pulped and water is added. Yeast is then added to the litchi juice and fermentation allowed to proceed, and (c) sparkling plum wine: plums preserved in sodium benzoate, a sugar concentration of 1.5%, a diammonium hydrogen phosphate concentration of 2% and the yeast *S. cerevisiae* strain UCD 595 are used to yield optimal results (Joshi et al. 1999); (k) *Brandy*: This refers to the distillate obtained by distillation of wine or any other fermented fruit juice or residue. Grape products are most commonly used, but apple, peach, plum, cashew apple, and apricot products can also be used. Various yeasts are involved in fermentation including *S. cerevisiae*, *S. capensis*, *S. ludwigii*, *S. rosei*, and *S. uvarum*. SO₂ is not used in order to prevent formation of sulfuric acid which would considerably lower the pH (Joshi et al. 1999).

6 SOY SAUCES

It is a dark-colored liquid that adds a meatlike and salty flavor as well as color to food. It is made by the hydrolysis of soybeans with or without wheat added.

(a) *Japanese Shoyu*: The molds *A. oryzae* or *A. soyae* are involved in fermentation. The yeasts *Saccharomyces rouxii*, *Torulopsis versatilis*, or *T. etchellsii* are also involved. Salted cooked soybeans are mixed with ground roasted wheat and the mixture is inoculated with *A. oryzae* seed culture. Pure cultures of *Pediococcus soyae* and *S. rouxii* are added at the start and again one month later. Fermentation proceeds at ambient temperature for a period of up to 3 years. Filtration, pasteurization, and bottling are then carried out (Yokotsuka 1977); (b) *Korean soy sauce Kanjang*: Molds of the genera *Aspergillus*, *Mucor*, *Penicillium*, *Rhizopus*, and *Scoptariopsis* are involved in brine fermentation (Lee and Cho 1971). The bacteria *Bacillus subtilis*, *B. pumilis*, and *L. mesenteroides*, and the yeasts *Rhodotorula flava*, *S. rouxii*, and *Torulopsis dattila* are also present. Steamed soybeans are crushed and left to mold without addition of inoculum. Fermentation is allowed to proceed for a couple of weeks, before filtration, pasteurization, and bottling (Lee and Cho 1971); (c) *Chinese jiang*: Molds of the *Aspergillus* type overgrow soaked, steamed soybeans coated with wheat flour. Fermentation is carried out at high temperatures and in the presence of salt brine; (d) *Malaysian soy sauce Kicap*: Soybeans are boiled, mixed with wheat flour, spread on bamboo trays to allow fungal growth without addition of inoculum, transferred to earthenware jars, covered with salt brine and incubated in the sun. After 3 months of fermentation, the sauce can be extracted. Brine can be added to the remaining mash for further extractions. Sugar, molasses, caramel, monosodium glutamate, and benzoic acid may be added to the sauce. *A. oryzae*, *A. soyae*, *A. niger*, *A. flavus*, *Rhizopus* sp., and *Penicillium* sp. are involved in solid-substrate fermentation in soy sauce factories (Ong 1977); (e) *Indonesian soy sauce Kecap*: The molds *Aspergillus* and *Rhizopus* spp. and various

yeasts are involved in fermentation (Poesponegoro and Tanuwidjaja 1977).

7 FERMENTED SOYBEAN PASTES

The three soybean pastes described in the following are used in making soup or side dishes. Japanese Miso and Indonesian Tauco have greater economic significance than Korean Doenjang and Kochuzang.

(a) *Japanese Miso*: This is prepared by fermentation of soybeans, with or without addition of rice or barley, using *A. oryzae* or *A. soyae* and *S. rouxii* (Hesseltine and Shibasaki 1961); (b) *Korean Doenjang and Kochujang*: *A. oryzae*, *Mucor* sp., *Penicillium* sp., *Rhizopus* sp., *R. flava*, and *T. dattila* are some of the essential microorganisms used in fermentation (Chang et al. 1977); (c) *Indonesian Tauco*: *A. oryzae*, *Rhizopus oligosporus*, and *Hansenula* sp. are involved. Soaked soybeans are boiled, dehulled, washed, boiled again, and covered to encourage fungal growth. Alternatively, they are inoculated with ragi tempe and mixed with rice flour, and incubated for several days following the second boiling. They are then dried in the sun, put in salt brine, fermented for 3–4 weeks before the addition of palm sugar, cooked, then bottled or packed (Winarno et al. 1977).

8 LEGUME-BASED FERMENTED FOODS

Legumes are used for food production mainly in Asia. Fermentation improves digestibility of legumes by hydrolyzing proteins, and breaking down antinutritional molecules like trypsin inhibitors.

(a) *Tempe (Tempeh) Kedebe*: This is a fermented soybean-based food, popular with American vegetarians and also available in Canada, the West Indies, Holland, Indonesia, and Malaysia. It is supplied in the form of a white, moldy cake. The beans are cleaned, soaked, dehulled, partially cooked, drained, inoculated, packed in banana leaves or perforated plastic bags, and incubated for 2 days to produce tempe. A variety of fungi have been isolated from Malaysian tempe including various species of *Aspergillus*, *Mucor*, *Penicillium*, and *Rhizopus* by Yeoh and Merican (1977). In tempe of other origins bacteria such as *Bacillus* and *Micrococcus* sp. may also be present; (b) *Tempelike foods from broad beans and cowpeas*: *Rhizopus arrhizus* is used in the production of tempe products from broad beans. *R. oligosporus*, *R. oryzae*, and *R. arrhizus* are used for tempe products from cowpeas. Different *Rhizopus* species give products with different aromas and flavors (Djurtoft and Jensen 1977); (c) *Oncom (Ontjon)*: A mixed culture of microorganisms with *Rhizopus* or *Neurospora* species predominating is used to produce this cakelike product formed by fermenting peanut presscake. Peanut presscake is soaked, drained, crumbled, mixed thoroughly with solid waste from tapioca production, steamed, cooled and formed into flat cakes, inoculated with

molds, covered with banana leaves, and fermented at room temperature for about 2 days to form oncom (Fardiaz 1987); (d) *West African Dawadawa*: This is prepared by fermenting locust beans and consumed mainly in West Africa. Yeasts, spore forming bacilli and lactic acid bacteria are involved in the fermentation. The pulp is removed from the seeds before they are boiled and then dehulled. The dehulled seeds are soaked, washed, cooked, spread on a tray, and covered with leaves then fermented for 2–3 days (Padmaja and George 1999). (e) *Chinese Chee-fan*: This is in solid form, prepared from soybean whey curd in China and eaten like cheese. *Mucor* spp. and *A. glauca* are involved in fermentation (Padmaja and George 1999); (f) *Chinese Meitauza*: This is Chinese soybean cake. It can be fried in oil or cooked with vegetables. *Actinomucor elegans* is the microorganism involved in fermentation (Padmaja and George 1999); (g) *Chinese Sufu (Tau-hu-yi)*: Soybean milk is made from ground soybeans strained through cheesecloth and boiled. CaSO_4 or MgSO_4 is added to induce curdling (protein coagulation). The cake remaining after pressing is known as tofu. Sufu is a highly flavored creamy bean paste prepared by growing soybean curd with *Actinomucor*, *Mucor*, or *Rhizopus* species and fermenting the curd in a salt brine/rice wine mixture. Red sufu is colored with a derivative from the culture of *Monascus purpureus* on rice while the white sufu is untreated (Wai 1929); (h) *Korean Meju*: This is soybean paste used for seasoning. *A. oryzae* and *Rhizopus* sp. are involved in fermentation (Padmaja and George 1999); (i) *Javan Bongkre*: This is coconut presscake popular in Central Java. *R. oligosporus* is involved in fermentation (Padmaja and George 1999). (j) *Indian Papadem*: This is solid crisp condiment made from black gram (*Phaseolus mungo*) in India. *Saccharomyces* sp. is involved (Padmaja and George 1999); (k) *Indian Warries*: Dehulled black gram grains are ground to a paste, spiced, and molded into small balls. These are then fermented for 4–10 days at room temperature and air-dried. The yeasts *Candida* spp., *Debaryomyces hansenii*, *H. anomala*, *Rhodotorula lactosa*, *S. cerevisiae*, and *Wingea roberstii* are involved in warri fermentation in addition to bacteria (Soni and Sandhu 1999); (l) *Indian Dosa*: This is a fried pancake-like staple food of South India prepared by fermenting a paste formed from rice and dehulled black gram. *S. cerevisiae* is the most predominant yeast involved in fermentation followed by *D. hansenii*, *H. anomala*, *Oosporidium margaritiferum*, *T. pullulans*, *Kluyveromyces marxianus*, *Candida kefir*, and *C. krusei*. Bacterial species belonging to *Leuconostoc*, *Bacillus*, and *Streptococcus* genera are also involved (Soni and Sandhu 1999); (m) *Yukiwari natto*: This is a kind of fermented whole soybean product made by mixing Itohiki natto with salt and rice koji. Rice koji, prepared by using *A. oryzae*, is the source of enzymes to hydrolyze the soybean components in fermentation, produced by *A. oryzae*. Itohiki natto is produced by inoculating soybeans which have been soaked, steamed and cooled

with *Bacillus natto*, and allowing fermentation to occur for about a day at 40–45°C (Kiuchi et al. 1976).

9 CEREAL-BASED FERMENTED FOOD

Cereals including wheat and rice form the largest class of food. Bread is the most commonly found cereal-based fermented food.

(a) *Chinese Minchin*: This is made from wheat gluten and used as a solid condiment. The fungal species involved in fermentation include *Aspergillus* sp., *Chadosporium* sp., *Fusarium syncephalastum*, and *Paecilomyces* sp. (Padmaja and George 1999); (b) *Chinese red rice (Anka)*: This is produced by fermenting rice with various strains of *M. purpureus* Went. It is used to color foods such as fish, rice wine, red soybean cheese, pickled vegetables, and salted meats. To make Anka, polished rice is washed, steamed, cooled, inoculated with *M. purpureas*, and allowed to ferment for a few weeks. Anka has been reported to be effective in treating indigestion and dysentery (Su and Wang 1977); (c) *Jalabies*: These are syrup-filled confectionery available in India, Nepal, and Pakistan made from wheat flour. *Saccharomyces bayanus* and bacteria are involved in fermentation (Padmaja and George 1999); (d) *Indian Kanji*: This is made from rice and carrots. It is a sour liquid added to vegetables. *H. anomala* is involved in fermentation (Padmaja and George 1999); (e) *Indian Torani*: This is prepared from rice and used as a seasoning for vegetables. *H. anomala*, *C. tropicalis*, *C. guilliermondii*, and *G. candidum* are involved in fermentation (Padmaja and George 1999). The prevalence of bacteria and yeasts in Indian fermented foods during different seasons varies. Yeasts such as *Candida vortiovaarai*, *C. krusei*, and *Kluyveromyces marxianus* are frequently present in the winter. *H. anomala*, *P. membranefaciens*, *S. cerevisiae*, and *T. beigelii* are present in both summer and winter (Soni and Sandhu 1999).

10 MIXED FERMENTED FOOD

They may give a higher protein content and a better balanced ratio of amino acids.

Nigerian Burukutu: This is a creamy drink made from sorghum and cassava. *Candida* sp. and *S. cerevisiae* are involved in addition to lactic acid bacteria (Padmaja and George 1999).

11 TUBER CROP-BASED FERMENTED FOOD

Tuber crops include cassava, yams, taros, potatoes, and sweet potatoes. With the exception of cassava, tuber crops keep well and hence not many fermented products are prepared.

(a) *African Fufu*: This is made from cassava roots and eaten with soup, sauce, or stew. Peeled cassava roots are

washed, cut up, soaked in water to release HCN into water, disintegrated, and sieved. The filtered starchy particles are allowed to settle and collected, rolled into balls, cooked, and formed into a paste called fufu. *S. cerevisiae* and various bacteria are involved in the fermentation (Padmaja and George 1999); (b) *West African Gari*: This is made from cassava roots and eaten as a staple food. *Candida* sp. and bacteria are involved in fermentation. Roots are fermented, broken up, sun-dried, milled into flour and made into a paste with boiling water before consumption (Padmaja and George 1999). (c) *Nigerian Lafun*: This is a fine, powdery cassava product. *Candida* sp. and bacteria are involved in fermentation (Padmaja and George 1999); (d) *Indonesian Tape*: This is a staple food made from cassava roots. To make Tape, cassava roots are peeled, cut up, boiled to soften, cooled, spread in trays, inoculated, covered with banana leaves, and fermented for a couple of days. The microorganisms involved in fermentation include *S. cerevisiae*, *H. anomala*, *R. oryzae*, *Mucor* sp., and *Endomycopsis fibuliger* (Padmaja and George 1999); (e) *Hawaiian Poi*: This is made from taro corms. It is a semisolid dish served with fish or meat. Lactobacilli and *Candida vini* and *G. candidum* are involved in the fermentation process which is carried out for 1–3 days at room temperature (Padmaja and George 1999).

12 FERMENTED FISH PRODUCTS

Fermented fish products, with their characteristic flavors, introduce variety to the South-East Asian diet.

(a) *Japanese katsuobushi*: This is made from fish and used for seasoning. *A. glaucus* is involved in the fermentation (Graikoski 1973); (b) *Cambodian Phaak or Mamchas*: This is a fermented paste produced from eviscerated salted fish. Glutinous rice pretreated with yeast is also added to the fish (Padmaja and George 1999); (c) *Vietnamese Nuoc-mam*: This is a brown liquid produced by fermentation of small marine or fresh water fish that are placed in earthenware vessels buried in the ground for several months. Bacteria and yeasts contribute to proteolysis and flavor. Enzymes from *A. oryzae* can be used for reduced fermentation time to increase yield of nuoc-mam (Richard 1959).

13 SALTED SEAFOODS

Seafoods including fish are nutritious and popular as food. However, they are highly perishable. Processes such as salting are used to preserve seafoods.

Korean Jeotkal: Fish such as herring and sardines, shrimps, cuttle fish, oysters, and clams are salted and stored to allow aging. Halophilic bacteria exhibiting protease, RNA depolymerase, and 5'-phosphodiesterase activities play a role. *Saccharomyces* and *Torulopsis* become dominant about 40 days after aging when halophytic bacteria disappear (Lee et al. 1977). Differences in flavor between different types of jeotkal are attributed to variations in the content of free amino acids

and 5'-mononucleotides. The protein content of jeotkal is higher than that in vegetable foods while the vitamin content is dependent on the type of jeotkal.

14 FERMENTED MEAT PRODUCTS (SAUSAGES)

The starter culture used in meat fermentation may have bacteria, the yeasts *Deboryomyces hansanii* and *Candida famata* and the fungi *Penicillium chrysogenum* and *P. nalgiovense*. Yeasts encourage color development and improve aroma in sausages. Molds contribute to the characteristic aroma. During fermentation, the fall in pH due to glycolysis by lactic acid bacteria helps to preserve the fermented product and inhibit the growth of pathogenic microorganisms, and the production of nitric oxide due to activity of the nitrate and nitrite reducing bacteria results in the production of nitrosomyoglobin that accounts for the odor of the meat product. During ripening, proteolytic activity due to enzymes in meat and bacterial starter cultures, and lipolytic enzymes in molds and yeasts lead to products which may contribute to flavor (Hammes and Knauf 1994).

15 CONCLUSIONS

Fermented foods and drinks play a substantial dietary role in people living in affluent nations as well as the people of developing countries. These foods and drinks are found in supermarkets and in wet markets. The prices of these products vary markedly: some wines may be expensive while some bean products are much cheaper. Man wisely exploited fungi in the production of tasty foods and drinks well before the advent of modern biotechnology. Today some of these fermentation procedures have developed into lucrative enterprises. Some of the fermented products, e.g., red wine, reportedly have health-promoting effects. Many of the fermented products have been found to be aflatoxin-free. Nevertheless, a few incidents of poisoning after consumption of fermented food have been reported. It is essential that aflatoxin-free raw materials and nontoxic cultures be used for food fermentation.

Recent research on fermented food has focused in several areas including the effect of fermented foods on health. The anka mold, *Monascus anka*, contains the antioxidant dimeric acid (Aniya et al. 2000). The hypocholesterolemic effect of fermented dairy products and their mechanism of action have been reported (St-Onge et al. 2000). A principal flavor component of soy sauce, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-(2H) furanone is a potent anticarcinogen in mice (Nagahara et al. 1992). Both beneficial and harmful effects due to kombucha (tea fungus) ingestion in animal experiments and in humans have been described (Greenwalt et al. 2000).

Another area of recent research is the investigation of the chemical constituents and nutritive values of fermented foods.

The changes in the major components of kombucha during prolonged fermentation have been followed (Chen and Liu 2000). The nutritive value of the fermented Nigerian beverage, burukutu and the chemical changes have been examined (Odetokun 1997). The levels of ethyl carbamate in alcoholic beverages and fermented foods (bread and cheese) have been determined (Dennis et al. 1989). Microbial analyses have been conducted on fermented food and beverages (Cosentino et al. 2001). The control of food-borne pathogens during sufu fermentation has been investigated (Shi and Fung 2000). The stability of stored fermented foods e.g. gari has been studied (Sanni 1996). Hopefully all this research will help food fermentation become a thriving industry which will introduce more varieties of food and also improve human health.

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Production of Edible Fungi

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1 INTRODUCTION

The world of mushrooms, owing to their sudden appearance in numbers, groups, rings, bunches, and also in isolation as a single attractive and imposing structure, has fascinated the man since time immemorial and references are available in the scriptures of many ancient civilizations. Theophrastus (372–227 BC), the great Greek philosopher, wrote about food value of mushroom when the latter found its way in the royal dishes for Greek and Roman emperors. There are indications that mushroom existed long before the *Homo sapiens* appeared on Earth, as evident from the fossil records of the lower Cretaceous period, i.e., about 130 million years ago; it is assumed that the primitive man also consumed mushrooms. The collection and consumption of mushrooms from the wild is still a practice in many regions of the world but the scenario changed after successful artificial cultivation of mushrooms. Though the Chinese are reported to have cultivated some specialty mushrooms like *Auricularia*, *Flammulina* and *Lentinula* between 600–1000 AD but, undoubtedly, it was the artificial cultivation of the common button mushroom (*Agaricus bisporus*) in France around the year 1650 which transformed the world of mushroom production and consumption.

Not all mushrooms are edible, but some are highly poisonous. While edible fleshy fungi are called mushrooms, poisonous ones are termed 'toadstools.' It has been estimated that out of 10,000 species of fleshy fungi (Kendrick 1985) about half of them are edible (Chang 1993) and as many as 100 species are highly poisonous. Collection and consumption of wild mushrooms requires knowledge and adequate precaution. Rather it is one of the most important reasons for popularization of artificial cultivation of proven edible mushrooms.

Commercial production of edible fungi represents unique exploitation of the microbial technology wherein worthless wastes (agricultural, industrial, forestry, and household) are efficiently converted into nutritious food. Indoor cultivation of mushrooms utilizing the vertical space is the highest protein producer per unit area and time, almost 100 times more than the conventional agriculture and animal husbandry. It has promising scope to meet the worldwide food shortage, without undue pressure on land, for the human population increasing at an alarming rate of almost 2 lakh people per day. Of about 2000 edible fleshy fungi, 20 types are being artificially cultivated and about ten are being produced and marketed in sizeable quantities: the common button mushroom (*A. bisporus*), oyster mushroom (*Pleurotus* spp.), shiitake (*Lentinula edodes*), black ear mushroom (*Auricularia* spp.), paddy straw mushroom (*Volvariella volvacea*), winter mushroom (*Flammulina velutipes*), silver ear mushroom (*Tremella fuciformis*), nameko (*Pholiota nameko*), monkey head mushroom (*Hypsizygus marmoreus*) and two famous medicinal mushrooms namely Maitake (*Grifola frondosa*) and the Reishi (*Ganoderma lucidum*).

2 WORLD PRODUCTION OF MUSHROOMS

The rapid rate of development of mushroom production technology from a primitive cave culture in France to a high-tech industry during the last three centuries is a success story which has kept pace with the ever-increasing demand for this commodity and there is every reason to be optimistic about its further growth in the years to come (Rai and Verma 1997). From a meager 2 million tonnes in 1986, the world mushroom production has registered a 3-fold increase in a decade and was about six million tonnes in 1997, and five mushrooms, namely *A. bisporus*, *Pleurotus* spp., *V. volvacea*, *L. edodes*

and *Auricularia* spp., the so-called leaders, accounted for 82 per cent of the total mushroom production (Table 1).

It is clear that the button mushroom (*A. bisporus*) is still the leader contributing 31.8% to the total mushroom production but its share that was 56.2% in 1986 has decreased over the years. China is the biggest producer of *Lentinula*, *Pleurotus*, *Auricularia*, *Volvariella*, *Flammulina*, and *Tremella*. Indonesia and Thailand are the other major producers of *Volvariella*. Japan produces significant quantities of *Lentinula* and *Flammulina* and almost the whole quantity of *Hypsizygus* and *Grifola* produced in the world. *Agaricus* production is concentrated in three geographical regions—Europe, North America, and East Asia. In Europe, Netherlands, France, and UK; in America, USA and Canada, and in East Asia, besides the giant China, Indonesia and Taiwan are the other major producers of this mushroom. Asia accounts for major share of US imports. It is quite interesting that six countries called group of six or G-6 countries (USA, Germany, UK, France, Italy, and Canada) account for more than 80% of world consumption; per capita consumption in these countries is very high (2–3 Kg). One important indicator of demand for mushroom is the income level of populace in the G-6 nations. Rise in income level is a global phenomenon and the demand for mushrooms is expected to grow at a much faster rate.

3 NUTRITIONAL AND MEDICINAL VALUES OF MUSHROOMS

It is primarily the flavor and texture for which the mushrooms are devoured by the mankind, and scientific appreciation of their nutritional and medicinal attributes is a recent phenomenon. Mushrooms have, from nutrition point of view, a distinct place in human diet which otherwise consists of items either of plant or animal origin. Mushrooms are perhaps the only fungi deliberately and knowingly consumed by human beings, and they complement and supplement the human diet with various ingredients not encountered or deficient in food substances of plant and animal origin. Besides the attributes understood in the terms of conventional nutrition, unique chemical composition of mushrooms makes

them suitable for specific groups suffering with certain physiological disorders or ailments like obesity, diabetes, atherosclerosis, hypertension, hyperacidity, constipation, etc (Rai 1995; 1997).

Nutritional value of mushrooms has been reviewed by many workers (Chang and Miles 1989; Crisan and Sands 1978; Rai 1995). Only salient features will be briefly but critically described here. It is a fact that there are wide variations in the nutritional values reported for the same species by different workers (Beelman and Edwards 1989; Rai et al. 1988). However, certain generalizations do emerge. Owing to very high (90%) moisture content these are basically a low calorie food (25–35 cal per 100g fresh weight) and this fits in well in this era of healthy eating by reducing the intake of calories. However, fat content in mushrooms is very low, it is rich in polyunsaturated fatty acid and is cholesterol-free (Rai 1995). Though carbohydrates constitute the major fraction (50% of dry matter), they are not nutritionally significant as the chitin (fungus cellulose), a polymer of N-acetyl glucosamine, is the structural component of cell wall and constitutes the major fractions of the carbohydrates and fiber. Chitin-N is also reported to give inflated values of the protein content of mushrooms if estimated by quantifying nitrogen and multiplying by the usual factor of 6.25. The fiber content is high in all the mushrooms (10% of DW). Mushrooms, due to high quantity and quality of protein have been recognized by the FAO as food contributing to the protein nutrition of the countries depending largely on cereals. Expectedly, in the nutritional evaluation of mushrooms, proteins have been the focus of attention of the researchers, but wide variations in the values for protein content of the mushrooms have been reported (Beelman and Edwards 1989). Rai et al. (1988) determined protein content in seven *Pleurotus* species (Table 2) by various methods of protein determination and found that protein values obtained with Folin-Phenol method of Lowry were closest to $N \times 4.38$ values as suggested by Crisan and Sands (1978). In terms of protein quantity, mushrooms ranking below animal meats rank well above common vegetables and fruits. The quality of mushroom protein is far superior to the vegetable proteins and is as good as or slightly inferior to animal proteins. This is because all the essential

Table 1 World production of cultivated mushrooms

Mushroom	1986		1997	
	Fresh (× 1000 T)	(%)	Fresh (× 1000 T)	(%)
<i>A. bisporus</i>	1227	56.2	1956	31.8
<i>L. edodes</i>	341	14.4	1564	25.4
<i>Pleurotus</i> spp.	169	7.7	876	14.2
<i>Auricularia</i> spp.	119	5.5	485	7.9
<i>V. volvacea</i>	178	8.2	181	3.0
Others	175	8.0	1096	17.70
Total	2182	3763	6158	100.00

Source: Chang (1999).

Table 2 Protein content in *Pleurotus* spp. by various analytical methods

Species	Protein (N $\times$ 6.25)	Protein (N $\times$ 4.38)	Protein (Lowry)	Protein (dye-binding)
<i>P. eryngii</i>	3.10	2.18	2.18	2.12
<i>P. flabellatus</i>	2.80	1.97	2.01	1.89
<i>P. florida</i>	2.24	1.57	1.61	1.45
<i>P. membranaceus</i>	3.04	2.13	2.10	1.91
<i>P. ostreatus</i>	2.61	1.83	1.91	1.66
<i>P. sajor-caju</i>	3.47	2.43	2.51	2.20
<i>P. sapidus</i>	3.18	2.23	2.37	2.01

Source: Rai et al. (1988).

amino acids are present in mushrooms and, interestingly, most abundant is lysine (Table 3), in which cereals are deficient. It is therefore, suggested that mushrooms can supplement the cereal-based diet of the developing countries (Chang and Miles 1989; Rai 1995). Mushrooms are rich in B complex vitamins and special mention is to be made of the presence of folic acid and B₁₂. Though vitamin C is present, it is vulnerable to postharvest losses due to very high phenol oxidase activity (Rai and Saxena 1989b). Potassium, sodium, phosphorus, and magnesium are the predominant minerals. Iron is present in appreciable quantity in the available form but mushrooms are comparatively deficient in calcium.

Very significant pharmacological activities have been observed in some mushrooms; a billion-dollar industry exists for the medicinal mushrooms namely Reishi (*Ganoderma lucidum*), Maitake (*Grifola frondosa*), Shiitake (*L. edodes*), *Trametes versicolor*, etc. The medicinal mushrooms have been recently reviewed (Rai 1997; Wasser and Weis 1999) and will not be dealt with here.

4 PRODUCTION TECHNOLOGY OF EDIBLE FUNGI

Production of edible fungi or mushrooms involves many steps, mainly the following: (a) Raising and maintenance of

mushroom culture, (b) Seed or spawn preparation, (c) Substrate preparation, (d) Growing or cropping, and (e) Postharvest handling

While step (a) and (b) are more or less common and similar for most of the mushrooms, it is the substrate preparation, crop raising, and post harvest technology which vary with the type of mushroom. In this article after brief treatment of the step (a) and (b), the cultivation technology of the so-called five leaders mentioned earlier will be briefly described and reviewed. The successive steps of production of mushrooms are depicted in Figure 1.

4.1 Spawn Production

The term “spawn” is used for vegetative growth of mushroom mycelium on a suitable medium, to be used as inoculum or “seed” for the substrate in mushroom cultivation. Right kind and quality of spawn is very important in the cultivation of edible fungi. The technique of spawn preparation witnessed many developments before Sinden developed the currently used “grain spawn” on hard winter rye grain after addition of calcium salts and patented the process in 1932 and 1937. However, wheat grain is now most commonly used as the basal medium for spawn production. Kumar (1995) has described other substrates used for spawn.

Table 3 Essential amino acids (% crude protein) in edible mushrooms

Amino acid	<i>A. bisporus</i>	<i>P. sajor-caju</i>	<i>L. edodes</i>	<i>V. volvacea</i>
Leucine	7.5	7.0	7.9	4.5
Isoleucine	4.5	4.4	4.9	3.4
Valine	2.5	5.3	3.7	5.4
Tryptophan	2.0	1.2	Nd	1.5
Lysine	9.1	5.7	3.9	7.1
Threonine	5.5	5.0	5.9	3.5
Phenyl alanine	4.2	5.0	5.9	2.6
Methionine	0.9	1.8	1.9	1.1
Histidine	2.7	2.2	1.9	3.8
Cystine	1.0	1.2	Nd	3.2

Source: Bano and Rajarathnam (1982).

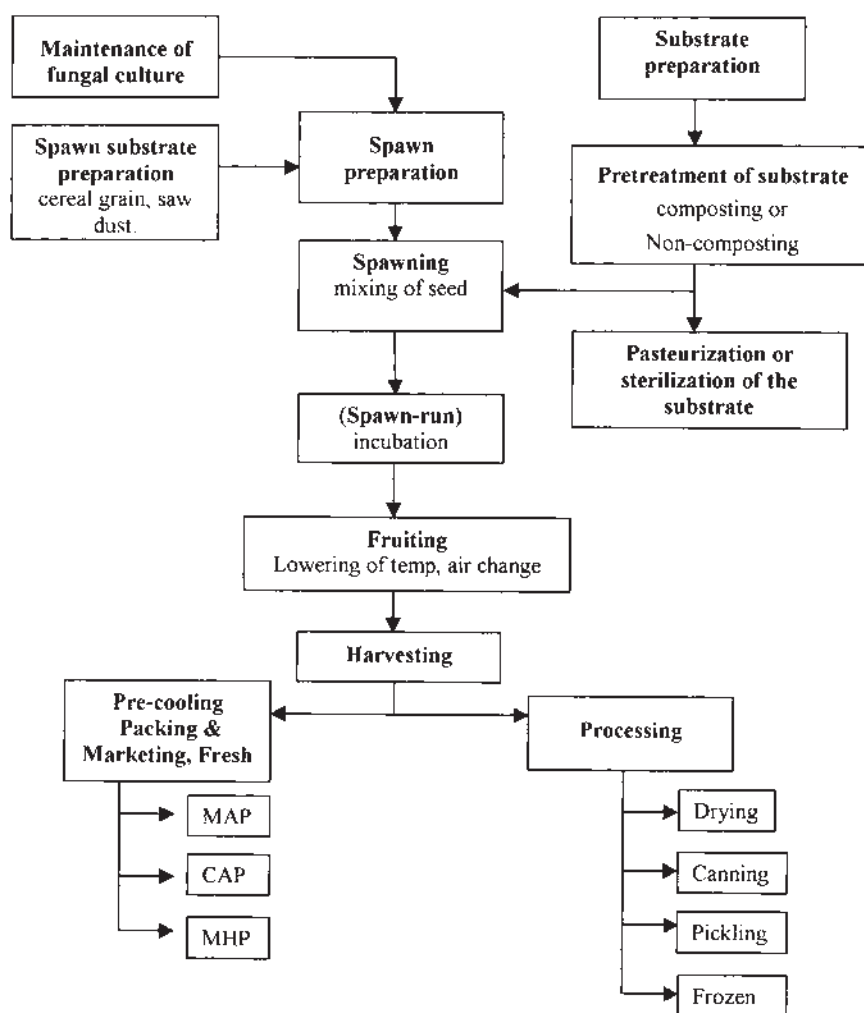


Figure 1 Major steps in mushroom production.

Often, failures to get satisfactory harvest are traced to spawn. If the spawn has not been made from a genetically suitable fruiting culture, or is too old and degenerated, the yield is likely to be poor. Ideal environmental conditions and management cannot compensate for the genetically inferior stock used to make spawn. In most parts of the world, there are specialized spawn manufacturing companies, which not only multiply the spawn but also are engaged in genetic improvement of mushroom germplasm; many high-yielding varieties/hybrids of mushrooms with superior quality attributes have been developed by them. The conventional techniques of growing mushroom mycelium on a sterilized substrate, often cereal grains, have been described by many authors (see Kumar 1995) but a revolution is taking place in the containers; it is no more glass bottles, but specially made polybags also called “breathing bags” which can withstand sterilization heat and allow limited gaseous exchange without chances

of microbial contamination have been developed. Some manufacturers have developed liquid spawn also. However, spawn for production of wood-decaying fungi is mostly prepared on wood chips and saw dust–cereal bran mixtures (Chang and Quimio 1982). Technique of spawn production has been described in detail by various workers (Chang and Miles 1989; Chang and Quimio 1982; Kumar 1995). Generally, wheat grains soaked in cold water are first boiled until these swell and become soft but do not rupture. After draining out excess water and surface drying in shade, calcium carbonate and calcium sulfate are added in such proportions to bring pH of the substrate in the range of 7–7.5. The substrate is then filled in glass bottles/polypropylene bags, cotton plugged, and autoclaved at 15 psi for 60–90 min. The substrate is inoculated with either pure culture for preparation of “mother spawn” or with mother spawn grains to prepare “planting spawn.”

4.2 Maintenance and Preservation of Fungal Cultures

Pure culture of edible fungi is prepared either by multispore culture or tissue culture; the former is suitable for obtaining fruiting cultures of *A. bisporus* but is not a suitable technique for heterothallic species. Tissue cultures derived from the stipe or pileus of the mushrooms, both homothallic as well as heterothallic species, can be used to raise fruiting cultures. For multispore culture, a healthy and mature fruitbody of the mushroom is first washed in sterile water, surface-sterilized with alcohol, and is placed on a spiral wire loop kept in sterile petriplate covered with a beaker. Mushroom sheds spores on petriplates from which a loopful of spores is transferred on suitable growth medium, generally malt extract agar in case of *A. bisporus*. Spores after germination give rise to multispore culture. In case of tissue culture, a piece from a suitable place of fruitbody is cut and after surface-sterilization, the piece is transferred onto sterile growth medium slants. Different mushrooms may require different growth medium and incubation temperatures; for example, *A. bisporus* grows best on malt extract agar medium between 24–28°C while *Volvariella* spp. are best maintained on potato dextrose agar medium at 35°C. It is desirable that the cultures are not maintained on the same type of medium in each subculturing for a long time. Degeneration of cultures or spawn, which refers to the loss of desired traits, survival, growth rate, and productivity, is not uncommon (Chang and Miles 1989; Stadelmann 1986) and has, of late, attracted the attention of the researchers to understand the reasons. Authentic pure cultures of mushrooms should preferably be obtained from the reputed mushroom germplasm banks. Now a days, mushroom strains of commercial importance are patented and thus free availability is restricted (Jong and Birmingham 1991).

Though the pure cultures of mushrooms, once raised or obtained as described above, are traditionally maintained by periodic subculturing and/or cold storing between 2–5°C, however, better long-term preservation methods of fungal cultures are advisable and practiced now, which are required for maintenance of vigor and genetic characteristics especially related with productivity and quality (Chang and Miles 1989). Frequent subculturing is not only time consuming but also costly and risky (Smith and Onions 1983). Other methods of preserving fungal cultures including mushrooms have been described (Jong 1989; Singh and Upadhyay 2002; Smith and Kolkowski 1996; Smith and Onions 1983), which include storage in mineral oil (paraffin wax), lyophilisation, cryopreservation at low temperatures (–70°C), in liquid nitrogen or in mechanical freezers. The choice of method depends on many factors like requirement, resources, cost, etc. It is advisable that each mushroom strain should be maintained by at least two different methods; liquid nitrogen and mineral oil preservation have been found highly suitable and are popular for preservation of mushroom cultures. Mushroom culture repositories/banks play a vital

role in the supply of pure and authentic cultures to the spawn production units. American Type Culture Collection in USA; International Mycological Institute in UK; Institute of Microbial Technology and National Research Centre for Mushroom in India are some of the reputed mushroom culture banks.

5 BUTTON MUSHROOM (*AGARICUS BISPORUS*)

Agaricus bisporus (Lange) Sing., popularly known as the white button mushroom, has the widest acceptability and still accounts for more than 30% of total production of all mushrooms. Limited quantities of *A. bitorquis*, a high temperature species, are also produced in some countries. Its cultivation technology has developed over the years from a primitive cave culture in France in the 16th century to a high-tech industry in America and Europe now. Still in many parts of the world, especially in developing Asian and African countries, sizeable quantities are being produced in low-cost structures like huts under the seasonal conditions. In some parts of the Europe, seasonal growing is done with arrangement for heating during the winters. Like any such venture, the production systems differ in the infrastructure, level of technology, automation, and mechanization but the basic principles and processes remain the same. The production technology of the white button mushroom (*A. bisporus*) has been described earlier by several authors (van Griensven 1988; Vedder 1978; Vijay and Gupta 1995). Most important aspect of the button mushroom production is the preparation of the selective growth medium, called compost, in which *Agaricus* mycelium thrives at the practical exclusion of other competing organisms.

5.1 Substrate (Compost) Preparation

Substrate preparation technique for the button mushroom has witnessed evolutionary changes over the years, from the long-method of composting to the current environment-friendly indoor composting. However, the intermediate short-method of composting, is still the most popular method all over the world.

5.1.1 Long Method of Composting

Long method of composting is the oldest method and now exists only in few pockets of the world mainly because of poor productivity, proneness to attack by the competitors, and also due to more time and labor consuming process (Vijay and Gupta 1995). This method is completely an outdoor process and takes about 28 days, though production of long-method compost in lesser duration has also been achieved. But the biomass loss in this process is very high (30–35%) and the quality as well as productivity is poor, besides the environmental problems it creates.

5.1.2 Short Method of Composting

Based upon the observations of Lambert that productive compost came from the regions of the pile having temperature between 50–60°C and adequate supply of oxygen, Sinden and Hauser (1950) developed the so-called short-method of composting mainly because it took lesser time than the long method. The concept and process was indeed a revolution in the cultivation of button mushroom. The short method of composting mainly consists of two phases: outdoor-composting for 10–12 days (Phase-I) followed by pasteurization and conditioning for 6–7 days inside specialized insulated structures, called tunnels.

Based upon the temperature conditions maintained inside the tunnel, Phase-II can be divided into two sub-phases: pasteurization (57–60°C for 6–8 h) and conditioning (45–48°C for 5–6 days). Importance of conditioning has been linked with the growth of desirable thermophilic microorganisms; pasteurization and conditioning are essential for achieving the selectivity in the compost. Short method of composting has many advantages over the long method: more compost per unit weight of the ingredients, higher productivity of mushrooms, less chances for pests and diseases, shorter duration and less environment pollution.

5.1.3 Indoor Composting

The problems of environmental pollution related with production of stinking gases associated with long as well as short methods of composting drew the attention of the researchers to evolve an alternative “clean” process. Because first time the work on such composting system started using completely indoor system, it was termed as indoor composting (Laborde 1992); other terms like environmentally-controlled composting, rapid indoor composting, and aerated rapid composting have also been used for this process. Based on the temperature conditions maintained inside the tunnel, the process could be divided into two categories, i.e., INRA method and Anglo-Dutch method. In the INRA method, which is popular in France, Italy, and Belgium, phase-I is carried out at constant temperature of 80°C for 2–3 days followed by phase-II at 50°C for 5–7 days (Laborde 1991). As very high temperature attained during phase-I of this method kills most of the microbes including the desirable thermophiles, reinoculation with mature compost or thermophilic fungi becomes necessary in this process. However, in the bunker system used nowadays this can be dispensed with. In the Anglo-Dutch method, a weeklong conditioning at 41°C follows a short pasteurization phase of 4–6 h at 60°C. The method has attained popularity in several European countries and Australia, and compost with high selectivity and substantial savings on raw materials (Miller 1997) is reported to be produced. Indoor-composting has many advantages even over the short-method of composting: takes lesser time, gives higher yield, is environment-friendly, and conforms to civic laws, lesser loss of raw materials and thus increased

end-product and above all, very high degree of selectivity (Miller 1997).

5.1.4 Growing or Cropping

Ready compost is seeded with spawn approximately at 0.5% on fresh weight basis after which the seeded substrate is either filled in polybags or in shelves and the temperature and humidity in the growing rooms are maintained at near 25–28°C and 90–95% RH respectively for 12–15 days for mycelial colonization of the substrate called spawn-running. Mushroom mycelium derives nutrition from the substrate by secreting an array of extracellular degradative enzymes capable of degrading cellulose, hemicellulose, lignin-humus complex, and several bacteriolytic and mycolytic enzymes. Once the compost is completely colonized, various types of supplements, often suitably treated proteinaceous materials like soybean meal is thoroughly mixed at 1% in the upper one third area of the substrate which is then layered with 4–5 cm of casing material. Depending upon the availability and suitability, various types of materials are used as casing in different regions of the world; peat is of course the substrate of the choice in the developed countries while farmyard manure (FYM) in various combinations with soil and other materials are used in many countries. Recently, choir peat (specially decomposed and processed coir pith dust) has found acceptability as casing material in many Asian countries where coconut plantations abound. Many authors (Hayes 1974; Kurtzman 1997; Vijay and Gupta 1995) have discussed materials, techniques, and the role of casing in cultivation of the button mushroom. Though there are varied observations and opinions on the importance of some properties of casing materials, e.g., pH, conductivity, bulk density, water holding capacity, and associated microflora, but that the button mushroom requiring a layering (casing) above the colonized compost for fruiting is a fact well-established. After casing, growing rooms are again maintained at high temperature (~25°C), humidity (~90%), and CO₂ (>5000–10000 ppm) for mycelium to colonize casing layer called case-run. “CaCing” (mixing of small quantity of colonized compost in casing material at the time of casing) and ruffling of partially colonized casing material few days before readying for fruiting are some of the improvisations practiced in high-tech mushroom production. After the complete colonization of the casing layer when white mycelium becomes visible between the clumps all over the casing surface, room temperature is lowered down to 16–18°C, fresh air is introduced/increased with slight decrease in the humidity (85% RH) i.e., the conditions not conducive for mycelial growth to continue. Under these “adverse” circumstances mycelial aggregation takes place to form pinheads or primordia which differentiate and develop into mushrooms (Figure 2). After growth to the desired size, mushrooms are handpicked or mechanically harvested. It is common observation that during the commercial cropping of *A. bisporus* and also of some other mushrooms, there is heavy and synchronous appearance, called “flushes,” of sporophores

at appropriate intervals with very little fruiting between the flushes called flush break. Fructification of mushrooms represents an interesting phenomenon to study the differentiation in multicellular eukaryotes (Rai and Saxena 1991).

Varied levels of mechanization in composting and cropping and automation especially in the environment control of the growing rooms have been introduced in many developed countries in case of the button mushroom. However, old manual system of seasonal growing in makeshift cropping rooms is still practiced in many developing countries to feed the domestic market.

6 SPECIALTY MUSHROOMS

“Specialty mushrooms” is a term given to a group of cultivated mushrooms which are less common in a particular area or country, but the term has been used to practically encompass all mushrooms other than the common button mushroom (*A. bisporus*). In the United States, the term “specialty mushrooms” is used to cover all mushrooms other than the button mushroom, which accounted for 90% of total production of 346188 MT there in 1993–1994 (Sharma 1997). In Japan, however, the situation is reverse to that in the United States where 90% of total production was of the so-called specialty mushrooms and button mushroom contributed only 10%. Therefore, from the Japanese perspective button mushroom could be termed as specialty mushroom. Be that as it may, the term specialty mushrooms is now well established by usage to represent all mushrooms

other than the button mushroom. Production and consumption of the specialty mushrooms are very popular in the East Asian countries namely China, Japan, Korea, Thailand, and Indonesia and is picking up fast in many American and European countries where these are considered as novelty.

The scope, importance, and cultivation technology of many specialty mushrooms has been covered briefly by various authors (Royse 1997; Sharma 1997; Stamets 2000) but this review will cover the production technology of the economically most significant specialty mushrooms namely, *Pleurotus*, *Lentinus*, *Volvariella*, and *Auricularia* which together accounted for 75% of the world production of specialty mushrooms in 1997 (Table 1).

6.1 *Pleurotus* spp. (Oyster Mushrooms)

Unlike most of the cultivated mushrooms, which represent one species, a group of species of the genus *Pleurotus* are commercially cultivated and referred commonly as oyster mushrooms. *Pleurotus ostreatus* (Jack. ex.Fr.) Kummer, is best known species among oyster mushrooms and the specific epithet “oyster” obviously refers to its Oyster-shell like appearance of the fruitbodies. *Pleurotus* spp. are most versatile of all the mushrooms, representing about fifteen species capable of growing over a wide range of temperature (5°C to 30°C) and on almost all the lignocellulosic wastes; *P. sajor-caju*, *P. florida*, *P. ostreatus*, and *P. flabellatus* are most popular commercial species. It is a primary rot fungi and can degrade moistened substrates directly and does not require precomposted substrates like secondary rot fungus, e.g.,



Figure 2 Button mushroom (*A. bisporus*) cultivation in polybags.

A. bisporus. Ease with which oyster mushrooms can be grown has manifested itself in the production statistics where the production of oyster mushroom registered 442% increase during the period 1980–1991 (Chang 1993).

Production of the oyster mushroom also involves the main steps of mushroom growing described earlier: selection of the substrate and its pretreatment, spawn preparation, spawning, incubation for spawn-run, and providing conditions for fruiting of mushrooms, i.e. cropping. Oyster mushrooms can be grown on a large variety of lignocellulosic wastes depending upon the availability and cost (Poppe 2000) but the cereal straws (wheat and paddy) are the most common substrates in many countries (Figure 3); cottonseed hulls are also popular in the United States. Substrate pretreatment is mainly aimed at moistening and so-called pasteurization/sterilization to give advantage to the mushroom mycelium and avoid contamination with moulds specially *Trichoderma* spp. Most commonly used pretreatments are hot water dip, pasteurization with steam, chemical pasteurization and steam sterilization (Jandaik 1997). Zadrazil and Dube (1992) have described a method for special substrate preparation for oyster mushroom. Pretreated substrate is mixed with grain spawn at 2 per cent by wet weight of the substrate and then filled in suitable containers, most commonly polybags. Bottle cultivation of the oyster mushroom is done in Japan. In some countries delayed-release nutrients, mostly formaldehyde-treated or polymer-coated soybean meal, is added in the substrate to increase the yield but the method involves risk of rise in bed temperature and contamination with moulds. Growth parameters for cultivation of *Pleurotus* differ from species to species, especially temperature requirement and

general parameters and have been described by Jandaik (1997). There are wide variations among the growers about the method and style of opening of the containers for fruiting: complete removal of polycover, only top open like Japanese method of bottle cultivation, and slashes or holes in polycover. Average commercial yields obtained are 1 ton fresh weight of mushrooms per ton of dry weight of the substrate. One peculiar and serious problem with the cultivation of the oyster mushroom is spore allergy, which sometimes develops among workers for which facemasks are generally used during the operations.

6.2 *Lentinula edodes* (Shiitake)

Shiitake is the second most important commercial mushroom; it contributed 25.4% of total mushroom production in 1997 (Table 1). Of late, production of this mushroom has become very popular due to not only its unique taste and flavor but also its unique medicinal properties, such as antitumor, hypocholesterolemic, and antiviral properties; Lentinan, a polysaccharide, is now an established immunomodulator (Mizuno 1995); Though Japan is the leading producer of shiitake its cultivation first started in China near 1100 AD, and the technology was perhaps passed on to Japanese growers by the Chinese.

Cultivation technology of the *L. edodes* has been described in detail by various authors (Harris 1986; Royse 2001). Traditionally shiitake has been grown on natural logs of various species of trees but currently oak (*Quercus*) logs are most popular. The type, size, and quality of logs used have



Figure 3 *Pleurotus florida* on wheat straw blocks.

been described by Royse (2001). Generally, logs of 7–15 cm dia are cut into 1 m lengths. Holes are then drilled; one row of holes is drilled for each 2.5 cm of log diameter and are evenly spaced length-wise every 15 cm along the row. Holes are plugged with wood piece spawn or sawdust spawn, and then finally sealed with hot wax; plug spawn is however preferred for varied reasons. Spawn run may take 6–18 months which depends upon many factors. Logs, after the spawn-run, are transferred to a growing yard, which should be cooler and humid than the spawn-run area. One interesting treatment given to induce fruiting in logs is “shocking treatment” where logs are banged with a hammer or dropped on end (Chang and Miles 1989). Production is very good during the spring and fall. Some growers, however, use green houses for winter production when the prices are considerably higher. In the green house cultivation technology, logs are generally soaked in water and vibrated mechanically prior to keeping in the houses. After taking the first flush, logs are reincubated for about 3 months and the process is repeated up to five times. Yields obtained from log system may be as high as 33%; best production occurs in second and third years. Shiitake production drops and is no longer possible after the bark is lost.

To make the shiitake cultivation more environment-friendly, synthetic log production system was developed where sawdust is the main ingredient; however, straw and corncobs are also used. Basal ingredients are supplemented with some starchy substance like cereal brans, maize, and some chemicals like calcium carbonate, calcium sulfate

(gypsum), and sugar. Ingredients are mixed in a mixer and moistened to a level of 60%. Moistened substrate is generally filled in polypropylene bags (2.5 Kg). Little holes are either made in the polybags or special breather patch made of micro-porous plastic is preprovided. The bags are then sterilized for 2 h at 121°C in large autoclaves and, after cooling, seeded with spawn. The bags after heat sealing are shaken to evenly distribute the spawn; sawdust spawn or cereal grain spawn is used in this system. Spawn-run at 21°C with 4 h of light per day takes 18–23 days for optimum growth. Colonized blocks are taken out by slicing and peeling off the polycover and kept for 4 weeks in the environment conducive for browning of the exterior surface i.e., at temperature of about 19°C and 2000–3000 ppm CO₂ and are watered once daily; and humidification may also be resorted to. As the browning process nears completion, pinheads start to form about 1–2 mm beneath the surface. Primordia development is stimulated by soaking the blocks in cool water (12°C) for 3–4 h; soaking is required for second and third flushes also. Mushrooms are ready for harvesting approximately after 7–11 days of soaking (Figure 4). Shiitake are harvested by gentle twisting by hand and stem cut with sharp knife like that for button mushroom. After harvesting, blocks are soaked again for 12 h, which may be 18 h in third soaking; flush breaks in shiitake are 16–20 days long. The total production cycle on synthetic logs is just 3–4 months and biological efficiency achieved is also very high (75–125%) as against cycle of 5–6 years and B.E. of 33% in natural log cultivation (Royse 2001).



Figure 4 *Lentinula edodes* on synthetic (sawdust) block (experimental crop).

6.3 *Volvariella* spp. (Paddy Straw Mushroom)

Volvariella is a mushroom of the tropics and subtropics; it grows at a relatively high temperature of around 35°C. This is a very fast growing mushroom, it takes about 10 days from spawning to first harvesting, is easiest to cultivate with a cropping cycle of 3 weeks but does not give good economic yields and its shelf-life is poorest of all mushrooms. Understandably, its production in 1997 was only 0.18 million tonnes and it contributed only 3% to the world mushroom production while its share was 4.6% in 1986. Nevertheless, its significance lies in East Asian countries where staple food is rice and paddy straw can be utilized for growing this mushroom mostly for self-consumption or trade in the domestic market. Commonly cultivated species are *V. volvacea* and *V. bombycina*.

There are two commercial substrates for growing this mushroom: traditional paddy straw bundles made into beds and the other involves the use of cotton waste compost after a short period of fermentation. The latter has many advantages (Quimio 1993; Quimio et al. 1990). While outdoor seasonal farming is still done on paddy straw by small growers, modern indoor cultivation is done on fermented cotton waste. Grain (wheat, sorghum) or cereal straw spawn is used; however, cotton waste + tea leaves spawn has been found superior (Chang 1982). Several techniques have been used for the cultivation of *V. volvacea* in the tropics, which thrives in the temperature range of 30–35°C and R.H. of 75–85% (Quimio et al. 1990). However, the traditional method of cultivation in South-East Asia is on straw beds, both outdoor and indoor,

while the more sophisticated indoor technique is preferred for industrial scale production on cotton waste compost; the latter gives higher yield but is capital-intensive process. While the traditional paddy straw cultivation has been described in detail by several authors (Chang 1982; Khanna 1997; Quimio et al. 1990), modern indoor cultivation technology under controlled conditions on cotton waste compost will be dealt here (Chang 1982; Quimio 1993). Since 1973, straw mushroom has been cultivated completely on cotton waste compost in controlled conditions in Hong Kong and the technology is now being practiced in Thailand, Indonesia, Vietnam, Singapore, and some parts of Malaysia and Philippines. It takes about 4 days to make compost of cotton waste mixed with rice bran and water. Compost is filled (10 cm) on beds in a plastic mushroom house fitted with blower and polyduct and pasteurized with live steam for 2 h at 60–62°C, conditioned at 50–52°C for 8 h and then allowed to cool to 34–36°C suitable for spawning. The amount of spawn used is 1.4% of the dry weight of compost or 0.4% of wet weight. Full growth is achieved within 3–4 days at 32–34°C of room temperature. During spawn-running no water and light is needed and a little ventilation is provided. Then white light is provided with fluorescent lamps and fresh air is introduced. After removal of plastic sheets and sprinkling with water, growth of other fungi and actinomycetes retards while *V. volvacea* continues to grow. On the fifth day after spawning primordia appear, which take about 4 days to reach the harvesting stage. Straw mushrooms are not allowed to grow to umbrella like structures but are picked for marketing at the stage before volva enclosing the cap breaks or just after



Figure 5 Proper stage for harvesting of *V. volvacea*.

rupture; the former is “button stage” and the latter is “egg stage” (Figure 5). The first flush usually lasts for 4–5 days; four days later second flush starts but yield is just 10% of the first flush. For all purposes, only one flush is commercially taken and in this way, one mushroom house can give two crops a month or at least three crops every two months. The shelf life of *Volvariella* is very short; it liquefies even at 4°C and also at high temperature. While canning is done at the industrial scale it is generally traded fresh or dried by small farmers in the developing countries.

6.4 *Auricularia* spp. (Wood Ear Mushroom)

The species of *Auricularia*, commonly known as wood ear mushroom, are morphologically and, above all, texturally quite distinct from other mushrooms. With typical ear like morphology with cartilaginous texture and gelatinous surface, these are liked as well as disliked at the same time by different people. This mushroom is very popular in China and Southeast Asia but does not seem to attract western consumers. It has been reported to possess many medicinal attributes: treatment of piles, sore throat, anemia and hypocholesterolemic effect (Quimio et al. 1990; Royse 1997). Out of about 10 recognized species of *Auricularia* two main commercially cultivated species are *A. auricula* and *A. polytricha*, the former is thin and light coloured while the latter is the thicker, longer, hairy, and darker. *A. fuscusuccinea* is also produced on a limited scale. Thailand and Taiwan are the main producers of this mushroom.

Like shiitake (*L. edodes*), *Auricularia* are also produced on natural logs as well as on synthetic logs (sawdust medium), the latter is more popular, productive and profitable system. The cultivation of *Auricularia* on natural logs is popular in the areas where suitable trees are abundant. This mushroom, unlike *Lentinula*, is not fastidious about the type of tree species and almost any tree except pines can be used. A log diameter of 3–6 cm and length of 1 m is recommended. The time of season for log felling is similar to that for *Lentinula* log cultivation i.e., when leaves are just starting to dry in autumn then logs have sufficient sugar and moisture to support mycelial growth. Holes are made, spawned, and sealed like that for *Lentinula* and incubated (20–28°C) outdoors in the “laying yard” during which logs are turned upside down once a month. After about 2 months, logs are transferred to the cropping area which may be an open area in a forest or a green house or shed where logs are kept upright and frequently watered; ideal temperature for cropping is 15–25°C which of course depends upon the strain used. Mushrooms can be harvested about 30 days after exposure to cropping conditions. During the winters, production ceases but logs are kept protected for getting fruiting next spring by resuming heavy watering. The logs should continue to produce mushrooms year after year unless they become heavily contaminated with some wood decaying fungi.

In the synthetic log cultivation, the substrate consists of sawdust, cottonseed hulls, bran, and other cereal grains.

The substrate may be composted for up to 5 days or used directly after mixing. Compost is prepared by mixing and watering the ingredients (sawdust 78%, bran 20%; CaCO₃, 1%, sucrose 1%) in a large pile, which is turned twice at 2 days interval. Directly used substrate mainly has 93% cotton seed hulls, 5% wheat bran, and 1% each of sucrose and CaCO₃ which is moistened to a level of 60% and then filled into polypropylene bags and sterilized at 121°C for 60 min. Spawning is done in cooled substrate either with grain or sawdust spawn, manually or mechanically. Bags are incubated at 25 ± 2°C for 28–30 days for spawn-run preferably in dark area (<500lux). Temperature is then lowered to 20 ± 2°C and light intensity increased to 2000 lux to promote pinning for which 5–8 holes of 2.5 cm are cut in the bags for emergence and maturation of mushrooms. Biological efficiency of 100% and above has been achieved. In India this mushroom has been successfully cultivated on wheat straw supplemented with wheat bran (Figure 6) and B. E. of as high as 140% has been recorded (Bhandal and Mehta 1989).



Figure 6 *Auricularia polytricha* on wheat straw blocks (mark the slits).

7 POSTHARVEST TECHNOLOGY OF MUSHROOMS

Mushrooms are highly perishable and have short shelf life ranging from few hours to days depending upon the species and the storage environment. Weight loss, blackening, veil-opening, and microbial spoilage are the common undesirable postharvest changes besides many physiological and biochemical changes (Bano et al. 1997; Rai and Saxena 1989a; Saxena and Rai 1989). Mushrooms require utmost postharvest care like proper handling, packaging, precooling, cool-chain transport, and storage till consumed. Modified atmosphere packaging (MAP), controlled atmosphere packaging (CAP), and modified humidity packaging (MHP) of the button mushroom have been described by Anantheshwaran and Ghosh (1997). Individual Quick Frozen (IQF) mushrooms are gaining popularity with other frozen vegetables in the super markets.

Postharvest technology of mushrooms has been dealt in detail by many authors (Bano et al. 1997; Lal Kaushal and Sharma 1995; Saxena and Rai 1989). Mushrooms are delicate, contain 90% water, rich in phenolics and have very active phenol oxidase and protease activities. They lack protective covering of suberin or cuticle, unlike fresh fruits and vegetables. Postharvest physiological and biochemical changes do take place at very fast rate. Storage in package films, sodium alginate coating, chemical preservation, lactic acid fermentation, irradiation, steeping preservations have been attempted to varied levels of success for short-term preservation of mushrooms. But dehydration and canning are the most common forms of long-term storage and trade in mushrooms; while canning is the most common method for the button mushroom, drying is resorted to for storage and trade of all the specialty mushrooms. Button mushrooms are also freeze-dried in limited quantities. However, improvements in solar dehydration, cabinet tray drying, and tunnel drying technologies of mushrooms are needed to produce better product. Of late, many attempts have been made to produce value-added mushroom products like pickles, soup powder, ready-to-use mushroom curry, nuggets, biscuits, etc.; in future we may witness more of such products.

8 EDIBLE FUNGI AND RECYCLING OF THE WASTES/RESIDUES

There are very few wastes of lignocellulosic nature of agro-forestry origin, which can not be used for growing one or the other mushroom. Poppe (2000) has compiled the information on various agro-wastes, which have been used for growing mushrooms. Residues left after obtaining the main product (e.g., grains, cotton, sugar) pose problems of their disposal and many may prove to be environmental hazards. Cultivation of various edible fungi on these wastes represents one of the unique recycling mechanisms where hardly any residue is left unexploited in one form or the other. The substrate left after growing the mushrooms is though often called "spent mushroom substrate" (SMS), which is a misnomer and "post-mushroom substrate" (PMS) is a more appropriate term because it is not "spent" and can be further decomposed by new set of organisms. Many efforts have been made towards profitable utilization of the PMS. The subject has recently been reviewed (Ahlawat and Rai 2002; Levanon and Danai 1997a). PMS of one mushroom can be utilized for growing other mushroom after suitable treatments. The PMS of the oyster mushroom is fed to cattle as feed and the dung obtained is used for biogas production; slurry of biogas plant is used as casing material for the button mushroom and PMS of button mushroom including casing fraction is decomposed in composting pits to be used as manure for raising the crop which was used to grow the primary mushroom, in this case the oyster mushroom (Table 4). In Israel, the above system of complete recycling of wastes has been successfully developed and adopted by the growers (Levanon and Danai 1997b). PMS, especially of the button mushroom, after suitable treatment like re-composting, has proved very good manure for raising not only the horticultural crops but also the major crops; it can also be used for biogas production. As mentioned earlier, the PMS of the oyster mushroom is a very good cattle feed with improved protein content and digestibility. Post-mushroom substrates have also been used for reclamation of soil and bioremediation of soil and water (Ahlawat and Rai 2002). Cultivation of edible fungi has the above mentioned economic as well as environmental advantages; it represents the production of food (mushroom) feed, fuel

Table 4 Recycling mushroom substrates and wastes

Recycling course	Main product	Waste/byproduct
Cultivation of cotton/wheat	Grains/fibers	Wheat and cotton straw
Cultivation of oyster mushroom on straw	Oyster mushroom	Spent mushroom substrate (SMS)
SMS as cattle feed	Meat or milk	Manure
Manure for biogas production	Biogas (energy)	Slurry "Cabutz"
Cabutz as casing soil for button mushroom	Button mushroom	SMS
SMS composted for organic farming	Organic food crops	No further waste from SMS

Source: Levanon and Danai (1997a).

(biogas), and fertilizer (manure) by complete recycling of the lignocellulosic wastes of agriculture or forestry origin.

9 CONCLUSIONS

Mushroom cultivation is perhaps the most important microbial technology, after the yeast fermentation, in the economic terms. It promises to supply food with good quality protein produced from worthless lignocellulosic wastes of varied origins. In future, newer mushrooms are likely to be added to diversify the portfolio of the cultivated mushrooms and the production of the presently consumed mushrooms will increase with the genetic improvement of the strains and the advancements in the cultivation technology. Modern biotechnological tools and computer aided environmental control will break the yield barriers. Share of the specialty mushrooms including the medicinal mushrooms will rise further and mushroom cultivation is likely to spread all over the world. Newer methods of culture preservation, spawn and substrate preparation for the mushrooms are being worked upon. Modern developments in packaging, storage, transport, and processing including the value-addition of food items will be extended to mushrooms, which will further boost its consumption and production. Researches on the utilization of post-mushroom substrate have thrown light on immense usefulness of this venture for production of food, feed, fuel, and fertilizer from the wastes through mushroom cultivation.

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Mycoprotein and Related Microbial Protein Products

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1 HISTORICAL BACKGROUND

Microorganisms have long been used in the elaboration of foods for human consumption. Practically, every civilization has developed fermentation processes of one sort or another as the basis of their culinary traditions. However, the culture of microorganisms as a source of nourishment, rather than as food transformers, came with the advent of industrial fermentation technology. The first recognition of the value of surplus brewer's yeast as a feeding supplement for animals by Max Delbrück (1910) was rapidly followed by the production of yeast for food during the ensuing First World War. The novel microbial foods may not have appealed to the conservative human palate, but this drawback was vastly outweighed by the logistic advantage of a high productivity of aerobic fermentations in relatively compact installations. This overriding feature motivated military strategists to draw plans to produce large amounts of yeast and fungal biomass during, as well as between, the two World War periods. The cessation of open worldwide hostilities in the second half of the 20th century could have meant an end to interest in microbial protein production for food. However, new preoccupations regarding malnutrition in third world countries, or political and economic isolation, as in the case of the Soviet Union and China, maintained the scope of microbial fermentations as a practical solution for the production of food, at least in emergency situations.

Spectacular developments in the field of agriculture, later accompanied by important changes in international relations leading to the opening of the world food markets, overshadowed the worries of limited food supplies. This

rapid development rendered mass microbial food production uncompetitive against traditional agricultural food crops, which became readily available at comparatively lower prices. This fact also led to a progressive decline in Single Cell Protein research studies in the literature (Ugalde and Castrillo 2002).

Notwithstanding this decline, biomass production technologies evolved away from microbial protein as bulk food, towards new specialities, leading to a new revitalization in the field (Figure 1). Examples of this are a wide range of food flavors and aromas produced by Burns Philp Food and Fermentation group (<http://www.bpfoods.com>), and yeast products directed to human and animal consumption by Lallemand Inc (<http://www.lallemand.com>). Most notable example of the evolution of microbial biomass into new products is perhaps that led by Rank Hovis McDougall (RHM) in cooperation with Imperial Chemical Industries (ICI), founding Marlow Foods (now part of the AstraZeneca group), a company which started producing mycoprotein and related products under the commercial trademark Quorn™ (<http://www.quorn.com>). This company produces fungal biomass from *Fusarium venenatum* (formerly *F. graminearum*) in continuous culture and the resulting product is manipulated to achieve a texture and taste which are reminiscent of meat products, covering a market as a meat alternative for vegetarian formulations. The mycoprotein production process experienced an evolution of 20 years and an estimated Research and Development expenditure of \$40 million, before unrestricted clearance by the UK Ministry of Agriculture, Fisheries, and Foods was granted in 1985. Quorn products are currently the only fungal-based products

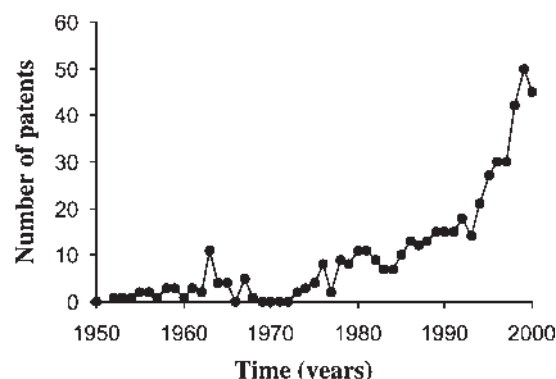


Figure 1 Number of food patents including “mycoprotein” or “yeast” in their title or abstract. Data from Esp@cenet network worldwide database (<http://ep.espacenet.com>).

exclusively directed at human consumption in the market. In this review, we intend to highlight the most important developments that have taken place in the production and use of mycoprotein, defined here as “microbial protein produced from microscopic fungi,” with mention of other specialities. The study includes relevant examples already present in the market and others, which have only been registered as patents. We will also conduct an exercise in determining what future lay ahead for mycoprotein and related products.

2 MYCOPROTEIN AS FOOD FOR HUMAN CONSUMPTION

Although a number of species has been reported to present favorable characteristics for the production of microbial

proteins and protein compounds (Halász and Lásztity 1991; Ugalde and Castrillo 2002), only a few of them are being actually used in industrial processes at the production scale (Table 1). The Quorn mycoprotein process is the main microbial protein production project directed to human consumption to date. Thus the Quorn project will be alluded in this review as the general example. Other projects will be specifically referred to.

2.1 Mycoprotein as Food

The microbial food product of the early 21st century does not base its market success solely on account of its protein content, as vegetable proteins sources are abundant in the market at competitive prices. The resemblance in texture to that of currently appreciated foods, and a bland taste and light color, which renders it susceptible to the addition of flavoring and coloring agents, are also a prerequisite. The filamentous nature of the organism, a feature, which was considered as a technical difficulty for production at first, was foreseen as an advantage in the case of Quorn, rendering the final product a resemblance to animal or fish meat. Rarer features, but by no means less important ones, such as those favoring health in normal humans, or indeed rendering beneficial effects in patients with high blood cholesterol levels or diabetes are certainly important, as will be discussed below.

2.1.1 Composition

Although fungal biomass can be considered principally as a source of protein, it also contains nucleic acids, carbohydrate cell wall material, lipids, minerals, and vitamins. These contributions are generally considered of little relevance, with

Table 1 Yeasts and filamentous fungi species accepted for production of protein compounds and food ingredients for the food industry

Yeasts	<i>S. cerevisiae</i> (baker's yeast)
	<i>S. cerevisiae</i> (brewer's yeast)
	<i>C. utilis</i> (Torula yeast)
	<i>K. marxianus</i> (formerly <i>K. fragilis</i> , <i>S. fragilis</i>)
	<i>K. marxianus</i> var. <i>lactis</i> (<i>K. lactis</i> , formerly <i>S. lactis</i>)
	<i>C. pseudotropicalis</i>
	<i>P. pastoris</i>
	<i>P. rhodozyma</i>
Filamentous fungi	<i>F. venenatum</i> (formerly <i>F. graminearum</i>) (Quorn products)
	<i>P. variotii</i> (Pekilo process, discontinued)
	<i>A. niger</i> ; <i>A. oryzae</i>
	<i>R. niveus</i> ; <i>R. oryzae</i>
	<i>Mucor</i> spp.
	<i>Streptomyces</i> spp.
	<i>P. roquefortii</i>

Data obtained from Halász and Lásztity (1991), Peppler (1983) and Centre for Food Safety and Applied Nutrition (CFSAN) and US Food and Drug Administration (FDA) (<http://www.cfsan.fda.gov>).

Table 2 Daily requirements (g) of essential amino acids for the human adult

Essential amino acids	FAO recommendation	Minimum
Phenylalanine	2.2	1.1
Methionine	2.2	1.1
Leucine	2.2	1.1
Valine	1.6	0.8
Lysine	1.6	0.8
Isoleucine	1.4	0.7
Threonine	1.0	0.5
Tryptophan	0.5	0.25
Total	12.7	6.35

Data retrieved from FAO (<http://www.fao.org>).

the exception of nucleic acids, which account for 10–15% (w/w) of the total nitrogen. Approximately 80% of total fungal nitrogen is composed of essential amino acids required for human growth and nutrition (Table 2). With reference to egg albumin, which is considered a perfectly balanced source of essential amino acids for human nutrition, mycoprotein presents a similar composition, although it is lower in sulfur containing amino acids. On the other hand, it is relatively rich in lysine and threonine if compared to other traditional protein sources of agricultural origin, such as wheat. A comparison with a wide range of protein sources is provided in Table 3.

2.1.2 Protein Value

The amino acid composition is only a theoretical indicator of the protein value of foods, since the degree of digestion and absorption of any one substance is determined by its susceptibility to be degraded and absorbed. The presence of inhibitors and multiple other factors in foods also modify their nutritional value. On the other hand, the digestive system of the organism in question is a key determinant in the

digestibility of various protein sources. Thus, empirical tests are usually required for feed evaluation, and the parameters used are: the total quantity of microbial nitrogen ingested (I), the nitrogen of faeces (F), and nitrogen in urine (U). From these parameters, Digestibility, Biological Value (BV), and Protein efficiency (PE) can be calculated. Thus, Digestibility (D) is the percentage of the total nitrogen consumed, which is absorbed from the digestive tract.

$$D = 100 \times (I - F) / I \quad (1)$$

Biological Value (BV) is the percentage of the total nitrogen assimilated, which is retained by the organism, taking into account the simultaneous loss of endogenous nitrogen through excretion in urine.

$$BV = 100 \times (I - [F + U]) / (I - F) \quad (2)$$

Protein Efficiency (PE) is the proportion of nitrogen retained when the protein under test is fed and compared with that retained when a reference protein, such as egg albumin, is fed.

Protein from yeast biomass such as *Candida utilis* has presented high digestibility values (81%), and BVs between 32 and 48%. Both parameters can be substantially increased when supplementation with 0.5% methionine is implemented (w/w, 90% in both cases). The BV of yeast has been estimated to be of 0.9, and methionine supplementation increases it to a value of 2.3 (Rivière 1977).

Quorn mycoprotein presents very high PE values, reaching 75% with respect to egg albumin. In experimental tests where mycoprotein was supplemented with 0.2% methionine, this value rose to 100%. Quorn products are not supplemented with methionine, but egg protein as explained below. Thus, mycoprotein could be used as a total replacement for the human diet (Trinci 1992, 1994; US Patent 5935841 1999; WO Patent 9117669 1991).

Toxicity testing of Quorn mycoprotein has shown that the product can be consumed as the sole source of protein on a continued basis, without any adverse effects. Given the unconventional nature of this product, the tests undertaken for

Table 3 Essential amino acid content (g per 100 g edible portion) of mycoprotein (Quorn), baker's yeast, egg (whole raw fresh), beef (ground, regular baked-medium), soybeans (mature seeds, raw) and wheat (Durum)

Amino acids	Myco protein	Baker's yeast	Egg	Beef	Soybeans	Wheat
Histidine	0.39	0.22	0.30	0.78	0.98	0.32
Isoleucine	0.57	0.48	0.68	1.02	1.77	0.53
Leucine	0.95	0.67	1.10	1.80	2.97	0.93
Lysine	0.91	0.69	0.90	1.89	2.43	0.30
Methionine	0.23	0.17	0.39	0.58	0.49	0.22
Phenylalanine	0.54	0.41	0.66	0.89	1.90	0.68
Tryptophan	0.18	0.11	0.15	0.26	0.53	0.18
Threonine	0.61	0.44	0.60	0.99	1.58	0.37
Valine	0.60	0.51	0.76	1.11	1.82	0.59

Data from US Department of Agriculture, Agricultural Research Service. USDA Nutrient Database for Standard Reference, Release 14 July 2001. Nutrient Data Laboratory (<http://www.nal.usda.gov/fnic/foodcomp>) and P. Collins, Marlow Foods, UK.

its approval were especially thorough, lasting ten years. Human trials involved 2500 people with no adverse effects. The mycoprotein product is approved for consumption in the European Union. The U.S. Food and Drug Administration is currently in the process of publishing an official response to the Full Food Additive Petition submitted by the manufacturers. This response will be made public in 2002 (FDA, <http://www.fda.gov>, GRAS Notice No. GRN 000091). Products containing mycoprotein have been on sale in the United States since January 2002.

2.1.3 Nucleic Acid Content

Nucleic acids are a necessary component of all cells, and are found in relatively high levels in rapidly dividing cells. Thus, the nucleic acid content of yeast (around 10% of dry weight) is approximately five times greater than in the average mammalian organ. When nucleic acids are ingested, they are first attacked in the duodenum by pancreatic nuclease. The resulting nucleotides are then attacked by nucleotidases in the intestine, resulting in nucleosides and phosphate. These in turn are further degraded to purine and pyrimidine bases. The degradation of purine bases in man results in the production of uric acid. Accumulation of uric acid beyond the excretion capacity of the kidney results in the formation of crystalline deposits in the joints and soft tissues, leading to goutlike manifestations and calculi in the urinary tract. Pyrimidines are degraded to orotic acid, the accumulation of which results in liver damage. The administration of foods of microbial origin is limited by the amount of nucleic acid contained within. The administration of 130 g of yeast daily for one week results in uric acid levels ranging between 4.8 and 8.3 mg/100 ml in human volunteers. Normal plasma levels of uric acid range between 2 and 7 mg/100 ml in males (Rivière 1977).

Quorn mycoprotein is obtained from a filamentous fungus which proliferates at slower rate than yeast (Trinci 1994; Ugalde and Castrillo 2002). Thus, the starting nucleic acid content subject to removal is also slightly lower (8–9%). The RNA content reduction of mycoprotein is further effected by a heat shock treatment that will be described below [see section “Mycoprotein production (Quorn products)”]. RNA levels are thus reduced well below the levels which limit consumption to 100 g per day per person (2% of dry weight), although this treatment also results in important losses in dry weight (Trinci 1992).

2.1.4 Texture

Another favorable feature which differentiates Quorn products is the advantage taken from the filamentous nature of the microorganism in product design. *Fusarium venenatum* A3/5 filaments are aligned in parallel by a specially designed mechanical process which renders the product a texture very similar to that of meat fibers once set in a light matrix of egg white protein and heated. The final product has a bland taste, light color which renders it susceptible to the addition of flavoring and coloring agents (Anderson et al. 1975).

2.1.5 Additional Functionalities

In addition to the nutritional effects, consumption of mycoprotein under both controlled and free-living studies has been shown to beneficially reduce total and low density lipoprotein (LDL) serum cholesterol levels. Studies by Homma et al. (1995), Turnbull et al. (1992), Udall et al. (1984) incorporating realistic amounts of the product concluded that mycoprotein consumption has a beneficial effect in serum lipid variables. Post-meal glycemia has been shown to be reduced after consumption of mycoprotein, by 13% with respect to controls. On the other hand, insulinemia is reduced by 19% thirty minutes after ingestion (Turnbull and Ward 1995). Finally, a mycoprotein lunch has a significant effect on the sensation of satiety, in ways that would help control the appetite of dieting patients, as proposed by Burley et al. (1993), Turnbull et al. (1993).

2.2 Mycoprotein Production (Quorn Products)

In order to ensure that Quorn is produced according to approved health regulations, the fermentation process is currently supported on glucose (nearly all of which is obtained from maize) and approved inorganic medium constituents and vitamins. However, the process may also be applied with various sources of starch as the carbon source

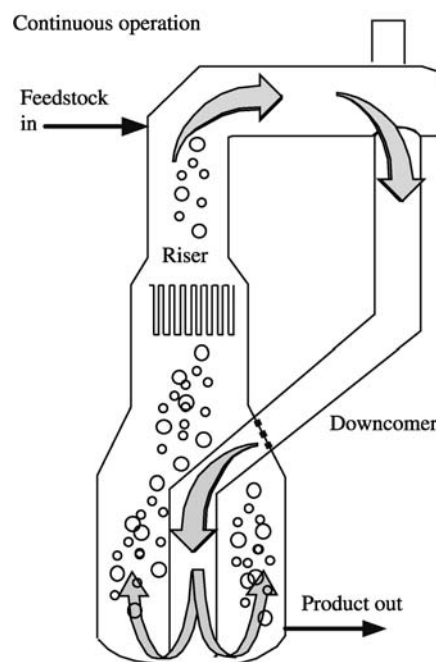


Figure 2 Diagrammatic representation of the Quorn™ air-lift fermenter used by Marlow Foods for the production of mycoprotein in continuous flow culture, (Courtesy: P. Collins, Marlow Foods, UK).

(Anderson et al. 1975; Steinkraus 1986). The production of *Fusarium venenatum* strain A3/5 takes place in turbidostat culture using air-lift fermenters of 155 m³ in volume and 50 m tall, weighing over 250 tons each. The Quorn fermenters are the largest operating air-lift fermentation facility in the world to date (Marlow Foods communication 2002).

Each fermenter operates as a loop where culture medium is circulating (Figure 2). As the liquid flows through the bottom of the loop, air is pumped in. Circulation is induced by a rising column of air bubbles providing good oxygen transfer conditions. This circulation is maintained due to mean density difference between riser and downcomer. The bottom of the downcomer hosts the glucose, biotin, and mineral salts intake. The nitrogen supply is delivered separately in the form of ammonia along with sterile air at the base of the riser. The supply of ammonia to the culture is regulated by a pH monitor set to give a culture pH of approximately 6.0. The dilution rate of the process ranges between 0.17 and 0.2 h⁻¹ and is operated so that glucose is always in excess and the fungus always grows at $\mu_{\max}$ at a biomass concentration of 10 to 15 g L⁻¹ (Trinci 1994). The culture is kept at a temperature of approximately 30°C by a heat exchanger set into the riser.

The dilution rate of the fermenter results in an output of 30 tons of liquid per hour. Harvesting by filtration and RNA reduction ensues. The harvested biomass (Figure 3) containing 8–9% (w/w) RNA is heated to 68°C for 25 min at a pH of 5–6. This results in the reduction of RNA to ca. 1% (w/w), at the expense of losing up to one third of the total mass, including dissolved salts, RNA, internal water, carbohydrates, and protein.

Studies on the incidence of the production phase duration on product cost and commercial viability had indicated that periods above 200 h operation can be necessary to result in consistent unit costs of production (Trilli 1977).

In principle, a continuous culture may run indefinitely, as long as contamination is kept under check. However, cultivation beyond 100 generations (about 400 h) of *F. venenatum* may result in the appearance of highly branched colonial mutants which can alter the texture of the final product (Wiebe and Trinci 1991; Wiebe et al. 1992; Wiebe et al. 1995).

The details behind the appearance of mutations in continuous culture, with specific reference to *F. venenatum* strain A3/5 have been studied by external research independent to the production process (Trinci 1992; Trinci 1994). From these studies, the authors obtained the conclusion that spontaneous mutant appearance may be managed by careful manipulation of the selective pressure imposed through culture conditions. The above-described strategies however have been successfully tested in laboratory conditions. These strategies do not conform to the industrial production process and are not necessarily used commercially (Marlow Foods communication 2002).

2.3 Related Products

Although there is a high variety of microbial protein formulations which can be used as food ingredients, apart from the major Quorn mycoprotein example a number of



Figure 3 Electron micrograph of *F. venenatum* A3/5 as collected from the outlet of the Quorn[™] fermenter, illustrating the filamentous nature of mycoprotein, (Courtesy: P. Collins, Marlow Foods, UK).

mycoprotein products are accepted and being used directly as food for human consumption (FDA, <http://www.fda.gov>; Halász and Lásztity 1991). Primary grown yeasts for use in human food are feed yeasts, inactive (nonfermentative) yeast cells, classified as "Primary Dried Yeasts" (category 96.1, Official Publication AAFCO 2002; Pepler 1983). The FDA admits the utilization of dried yeast from *S. cerevisiae*, *K. marxianus*, and dried torula yeast (*C. utilis*) for human consumption provided that the total folic acid content is not greater than 0.4 mg per g of yeast. Dried yeast derived from *S. cerevisiae* is also accepted for use in bakery products (Code of Federal Regulations, CFR. <http://www.access.gpo.gov/nara/cfr/index.html>; Title 21. Sections 172.325 and 172.896; Halász and Lásztity 1991).

Lallemand Inc (<http://www.lallemand.com>) produces inactive dry yeast (baker's yeast) as dough conditioner. Moreover, dry yeasts and filamentous fungi containing extra contents of minerals or vitamins are being used to meet specific nutritional requirements. Thus, Diamond V[®] and the Chinese Academy of Agriculture report the use of edible selenium enriched yeast and fungi (<http://www.diamondv.com>; CN Patent 1121531 1996), Lallemand Inc and Burns Philp (<http://www.bpfoods.com>) produce mineral and vitamin enriched yeasts for human nutrition, and Kohjin Co Ltd (<http://www.kohjin.co.jp>) and Universal Foods (subsidiary of Lesaffre, <http://www.lesaffre.com>) have registered patents for production of iron, selenium, glutathione, and zinc enriched yeasts (US Patent 4530846 1985; JP Patent 9248179 1999; JP Patent 2000279164 2000). Finally, an example of production of a cultured yeast product for human and animal consumption from *K. marxianus* has been registered by DMV USA (<http://www.dmv-international.com/dmvusa>) subsidiary of Campina International (<http://www.campina.com>) (US Patent 5486368 1996; EP0643765B1 2001).

3 MICROBIAL PROTEIN PRODUCTS AS FOOD INGREDIENTS

A multiple number of special products derived from baker's and brewer's yeast have been developed in order to complement the properties of other foods, rather than as major protein sources. Since the techniques for production of yeast are well established (Pepler and Stone 1976; Ward 1992), most of the new developments deal with post-production treatments of the biomass (Halász and Lásztity 1991). This aspect will be covered in detail in this chapter, therefore.

3.1 Active Formulations

The major microbial products used as feed additives are the two yeast categories approved to maintain their fermentative capacity, "active dry yeast" (96.2) and "yeast culture" (96.8) (Pepler 1983; Official Publication of Association of American Feed Control Officials 2002). Active dry yeasts

are used as starters in the baking industry and in other traditional fermentation industries where the leavening activity, and ability to ferment different raw materials, is necessary to improve storability, taste and flavor. At this moment, companies such as Beldem (<http://www.beldem.com>), subsidiary of the Puratos group, manufactures active dry yeast for use in bakery and Lesaffre group (<http://www.lesaffre.com>) and Lallemand Inc (<http://www.lallemand.com>) commercialize a wide range of sourdough starters and yeast products for the baking, brewing and wine industry.

Yeast culture is the only officially defined feed yeast ingredient which is not composed of isolated dried yeast cells only. Yeast culture consists mainly of growth medium with a moderate content in crude protein. It is being used as an aid to the ensiling process (lactic acid formation by naturally present bacteria), which increases feed value and improves the quality and palatability of the resulting silage, but the greater portion is used to fortify feeds and feed concentrates administered to livestock and poultry (Pepler and Stone 1976; Pepler 1983). As an example, Diamond V[®] produces active yeast cultures for fermenting liquid and cereal grain raw ingredients for animal feeds including dairy, beef, horse, poultry, aquaculture, and pet foods.

Yeast and fungi active cultures are also approved for production of commercial enzymes for the food industry. The FDA accepts the use of, among others: amyloglucosidase derived from *Rhizopus niveus*; carbohydrase from *R. oryzae*; lactase enzyme preparation from *K. lactis* and chymosin preparation from *K. lactis* or *A. niger*. New generally recognized as a safe (GRAS) fungi and yeasts are continuously under study with the objective of incorporation of new fungal enzymes into the food industry (Saxena et al. 2001; Wolf 1996).

3.2 Inactive Formulations

Inactivated biomass from yeasts and fungi can be processed into a variety of different valuable products serving as ingredients for animal and human consumption. Due to their nucleic acid content and cell wall compounds which may cause undesirable effects, the main strategies entail cell lysis and extraction of the components, mainly proteins, and separation and concentration for the production of concentrates and isolates. These can be used directly (e.g., Beldem produces inactive yeast for use in animal feed) or processed in order to get extracts, autolysates, and protein hydrolysates of specific functional properties.

Use of yeast protein concentrates and isolates in food processing is recommended in many scientific papers due to their favorable nutritional properties. However, due to the relative high cost of protein isolate production the use of these formulations is limited to meat products, meat, and milk substitutes.

Yeast and fungi autolysates and hydrolysates are obtained by hydrolysis of the proteic fraction. These can be used as natural flavors under the definition of the FDA and Code of

Federal Regulations (21 CFR 101.22(3)) and to improve the physical properties (texture, emulsifying properties) and nutritional value of food products (Halász and Lásztity 1991). Thus, yeast autolysates and hydrolysates are used as flavor enhancers in sausage, meat, and cheese manufacture (Halász and Lásztity 1991). Examples of these applications are: the production of *Aspergillus oryzae* extracts by Diamond V, used as a livestock feed, the autolyzed yeast flavor ingredients, Provesta[®] flavors from torula yeast, and Ohly[®] yeast extracts manufactured by Burns Philp (<http://www.bpfoods.com>) for use in different food systems, sauces, soups, marinades, and many other food types. Lallemand Inc, Lesaffre group, and Beldem also produce a complete range of yeast extracts for animal feed and as flavor enhancers for application in animal and human nutrition. At this moment, the studies are mainly focused on new developments and more tailored protein formulations whose acceptance by the consumer will depend on their functional characteristics.

4 SAFETY AND QUALITY CONTROL OF MICROBIAL PROTEIN PRODUCTS

The safety and acceptance of microbial protein products has to be strictly controlled, as in the case of all foods. The concepts underlying the acceptance and safety of novel protein sources including mycoprotein products were developed between 1970 and 1974 by the Protein Advisory Group of the United Nations (PAG), initially based on established toxicological experience. During the 70s the experience was accumulating with the incorporation of an increasing number of preparations of microbial origin, with more extensive trials in experimental and farm animals and preclinical and clinical testing (Scrimshaw 1985; Stringer 1985). The main concepts contemplated in these guidelines (Anonymous 1975; Anonymous 1983a,b,c; Scrimshaw 1985), which define the acceptance and quality control of microbial protein products are outlined below.

(1) GRAS status organisms. Generally recognized as safe (GRAS) status may be granted to an organism by qualified experts either on the grounds of: (a) scientific studies which confirm absence of toxic compounds and non-pathogenicity, and (b) in the case of a substance used prior to January 1, 1958, through experience based on common use in food (Code of Federal Regulations, 21 CFR 170.30, 182, 184, and 186). (2) Absence of health hazards and toxic compounds in the product. No living cells derived from the fermentation process (original strain or contaminants) can be present in the final product. Medium components that may be health hazards must also be absent. In case antifoams, detergents, or flocculants are used in the fermentation process, they have to comply minimum safety requirements or be removed completely (Anonymous 1983c; Scrimshaw 1985). Constraints are placed upon the heavy metal content of the final product. Typical maximum values are: fluoride (F) 150 ppm, lead (Pb) 5 ppm, arsenic (As) 2 ppm, mercury (Hg) 0.1 ppm,

although experience shows that the standard levels fit well below these limits. In products of fungal origin, chemical analysis of absence of mycotoxins is considered essential (Scrimshaw 1985; Stringer 1985). (3) Pathogenicity. The potential pathogenicity of a microorganism used for feeding, has been evaluated by the injection of the viable organism into the body cavity or body fluids of a mammalian species. In this way the nonpathogenicity of a large number of microorganisms (*S. cerevisiae*, *C. utilis*; *C. maltosa*, *C. lipolytica*, and *Torulopsis*) has been evaluated (Stringer 1985). (4) Integrity of the original strain. The maintenance of the integrity of the original strain and absence of undesirable contaminants has to be proved by specific microbiological and biochemical tests (Anonymous 1983c). (5) Continuous monitoring and control of process variables. To ensure quality and uniformity of the product the process variables (temperature, pH, aeration, cell concentration) have to be carefully monitored, and proof of such monitoring has to be presented. (6) Nucleic acid content. For animals or humans, nucleic acids coming from microbial protein sources added to the daily diet should not exceed 2 g per day (Anonymous 1975; Scrimshaw 1985). This restriction does not apply for nonprimate mammalian species or other vertebrates (Stringer 1985). (7) Toxicological and biochemical studies in animals. The required procedures for testing toxicology of microbial protein products are similar irrespective of whether the product is intended for animal and human feeding (Stringer 1985). First, the tests include short and long-term classical animal toxicology tests, with rodents and other target species (e.g. broiler chicken and pig) and the species that will finally consume the product in its diet (Stringer 1985). They consist of a complete battery of biochemical studies including effects on gross pathology, organ weight, and histopathological examination of the main organs and tissues (Anonymous 1983a). (8) Tolerance studies in humans. Clinical trials. Tolerance studies are used to determine the acceptance and the frequency of allergic and other undesirable reactions. The most common symptoms to be studied are gastrointestinal intolerance, skin rashes, and presence of other allergic reactions (Anonymous 1983b). The trials are conducted with a number of 50 or 100 individuals randomly assigned to experimental and control groups, stratified by sex, with careful report of all significant effects, symptoms, changes in mood, appetite, libido or sleep patterns, monitored in a diary along two sets of four-week study periods (Scrimshaw 1985; Udall et al. 1984). The official recommendations and guidelines of the PAG, part of them which have been briefly described here, are made specific in the form of official specifications for each microbial protein product, which are finally referred in the Code of Federal Regulations, Codex Alimentarius (<http://www.codexalimentarius.net>), AAFCO and other official publications, which are updated regularly.

The final control of the quality of the food product is regulated by specific official organizations and state food agencies through their respective directives. An example of this is the Food and Drug Administration (FDA, <http://www.fda.gov>) in collaboration with the Association

of American Feed Control Officials (AAFCO) and Centre for Food Safety and Applied Nutrition (CFSAN, <http://www.cfsan.fda.gov>). The food agencies have to establish among others, the Compliance Policy Guides, current Good Manufacturing Practices (cGMP), official methods and mechanisms of inspection and control to be used to evaluate the quality and safety of food products. As an example of the AAFCO and FDA policy, a direct-fed microbial product listed by the AAFCO Official Publication and not labeled or promoted with any therapeutic function will be regulated as a "food" and usually will not require FDA regulatory attention. However, a direct-fed microbial product with claims for disease cure, mitigation, treatment or prevention (e.g. a dietary product containing a specific bioactive compound) will be considered an "unapproved drug" and required for regulatory action, the initial proceeding being a warning letter (FDA, Compliance Policy Guide CPG 7126.41, Directed-Fed Microbial products). In a global perspective, taking into account the existing regulations and quality control procedures, the compactness and high degree of control achieved in production of microbial protein processes, which can be manufactured under controlled factory conditions in compliance with GMP requirements, may provide a high degree of safety to the consumer, against the uncertainties which regularly surround other food products.

5 CONCLUSIONS

Interest in mycoprotein and yeast biomass derived proteins has shifted since the mid twentieth century from a source of basic food, to specialty products which act as meat substitutes. This has been successfully achieved, and appears to be an expanding line. In addition, other products derived from biomass have been developed as food additives, which enhance the functional properties of other food-base formulations, such as flavor, emulsifying properties, and texture. This tendency is likely to be reinforced in the foreseeable future, as the risk of new food episodes emerging from uncontrolled industrial production practices, such as Bovine Spongiform Encephalopathy (BSE), variant Creutzfeldt-Jakob Disease (vCJD) or dioxin contaminations appear. In addition, some appreciated food stocks such as fish, appear to suffer increasing pressure, and limitations in catch quotas may in future bring in the development of mycoprotein-based fish substitutes. The increasing sophistication of mycoprotein-based food technology will not only facilitate the appearance of diverse specialty products in the near future. It will remain, as it did before, an important technology base for the fast production of foods in logistically demanding conditions. These include war conflicts, famine crisis, or cases of declared but uncontrolled contamination of foods. The validity of these technologies for the production of food in the long term should be kept into consideration, given the practical advantages derived from compactness of the

installations, the inherent safety of the fermentation technique, and the recycling potential of the by-products.

The use of genetically modified fungi for the production of foods is an inspiring subject, given the massive potential that DNA technologies have provided for the generation of new products. However, the introduction of this technology into traditional industries is currently limited by public perception. An increasing number of research studies are focusing on the development of genetically modified fungi for application in the food industries (Dequin 2001; MacCabe et al. 2002), but a very limited number of these studies have emerged in the form of new products or patents. Recently, a method of production of a fermented beverage using genetically modified yeast strains has been registered (US Patent 6326184 2001). However, the majority of food companies avoid the use of genetically modified raw materials or strains, and traditional processes dominate the market. The application of these techniques into real products is still considered as a long term process, which will depend on public acceptance (Uzogara 2000).

Our view is that there is a market for protein products of fungal origin, aimed at animal and human consumption, with the mycoprotein process (Quorn products) as a remarkable example. The success of the Quorn myco-protein process is determined by the product concept, where the business idea determines the design and development of process and product. Similar examples, applied to the production of new protein products from wild type and recombinant GRAS strains (Wolf 1996) will result in new processes and high value food products in the future.

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Genetic Variability of Yeast in Wine Fermentation

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1 INTRODUCTION

The first use of yeast for winemaking is lost in the dawn of the first agricultural civilizations. There are reports of wine-making as far as 7400 years ago. Until middle of the last millennium, wines were mainly produced around the Mediterranean Sea and the Caucasus. Since then, winemaking spread with the European colonizers throughout the temperate regions of the world (Pretorius 2000). Until 1863, must fermentation was not well understood from a microbiological point of view. In that year, Pasteur showed that a living microorganism, the yeast, was responsible for the biotransformation of the sugar present in the must into ethanol and CO₂. Although many genera and species of yeasts are found in the musts, the genus *Saccharomyces*, and mainly the species *S. cerevisiae*, is responsible for its biotransformation. Because of this, *S. cerevisiae* is referred as “the wine yeast” (Pretorius 2000).

2 YEAST BIODIVERSITY IN WINERIES

2.1 Yeast Species Diversity During Vinification

All the investigations carried out on grape surfaces by direct isolation (without enrichment) have constantly shown that *Hanseniaspora uvarum* (anamorph: *Kloeckera apiculata*) is always the predominant inhabitant of the grape surface (75% of the cells); this numerical supremacy may explain its initial domination in natural fermentations. *Metschnikowia*

pulcherrima is often present, followed by a group of film-forming yeasts (*Pichia anomala*) or pigmented species (*Rhodotorula* sp.). In a general study of yeasts isolated from grapes, however, it was noticed that the profile of yeast species may also vary from region to region (Martini and Vaughan-Martini 1990). Numerous factors affect the total yeast population and the relative proportions of individual species on the grapes. These factors include climatological conditions, the grape variety and the degree of maturity at harvest, the use of fungicides and the physical damage of the grapes (Fleet and Heard 1992). The yeast diversity found in wine-producing regions is strongly related to the quality and organoleptic characteristics of the wine produced from one year to another. However, the most significant finding was that *S. cerevisiae* is practically absent from grapes and vineyard soils (Martini and Vaughan-Martini 1990). The presence or absence of *S. cerevisiae* on grapes is the subject of some debate. Some authors propose that this species is a “natural” organism present in plant fruits (Mortimer and Polsinelli 1999; Sniegowski et al. 2002). Others argue that it is an artificial species that originated from the hybridization of other *Saccharomyces* species, and which then evolved over several centuries to become fully adapted to man-made environments such as wineries (Martini 1993). Finally, some other authors postulate that *S. cerevisiae* is a domesticated species that originated from its closest relative, *S. paradoxus*, a wild species found all around the world that is associated with insects, tree exudates, and fermenting plant extracts (Naumov 1996).

2.2 Enzyme Activities of Non-*Saccharomyces* Wine Yeasts

With respect to the role played by these non-*Saccharomyces* species in wine quality, they are known to have the capability to improve the wine aroma (Charoenchai et al. 1997; Esteve-Zarzoso et al. 1998). The available aromas in the grape impart and define the characteristics and the final quality of wine. Terpenic compounds account for most of these aromas. Grape processing liberates small quantities of aromatic terpenols; however, odorless precursors in the grape present a large, untapped reserve for wine aromas. Not only is the aroma an important quality factor in wine but also the intensity of the color is another very important quality factor in red wine, where anthocyanins are the main pigments. Various enzyme activities can improve the process of winemaking and enhance wine quality. The yeasts involved in wine making could be important producers of these enzymes (for a review see Table 1).

3 MOLECULAR METHODS: INTER- AND INTRA-SPECIFIC VARIABILITIES

The use of active dry yeasts is of particular interest to the wine industry, since the sensory properties of the final product vary considerably from one year to another depending of the microbial flora present on the grapes (Querol et al. 1990). It is generally assumed that indigenous yeasts are suppressed by the starter; however, different studies show that indigenous yeasts can still participate in the fermentation (Schütz and Gafner 1993; Querol et al. 1992a), although an implantation of only 50% was observed when fermentations were conducted by some commercial strains (Esteve-Zarzoso et al. 2000). For these reasons, rapid and simple methods for the routine verification of yeast strain present in fermentations would be useful to check the implantation of the starter.

Classical taxonomy is based predominantly on phenotypic characteristics, such as metabolic and/or cell morphology (Kurtzman and Fell 1998). However, nutritional characteristics have been shown to be highly variable as well as mutable, and genetic crosses have linked the characteristics to one or only a few genes (Petersen et al. 2000), which in some

cases could lead to an incorrect classification of a species or a false identification of strains. Methods based on the analysis of total cell proteins (Guillamón et al. 1993) and long-chain fatty acids using gas chromatography have been used (Augustyn et al. 1991), but their reproducibility is questionable because they depend on the physiological state of the yeast cells (Golden et al. 1994).

Recent progress in molecular biology has led to the development of new techniques for yeast identification and characterization (see Table 2). Most of these techniques are useless for the routine identification of yeasts and, most importantly, there is no available database to identify a large number of species. In addition, most of these techniques were applied to characterize only a few genera, and cannot be considered as a general method for yeast identification. In the next sections, we review those methods that have been preferably applied in recent years and their application to the rapid identification or specific characterization of wine yeasts in industrial practice.

3.1 Methods for Yeast Identification

The specificity of nucleic acid sequences has prompted the development of several methods for rapid species identification. The comparison of RNA (rRNA) and its template ribosomal DNA (rDNA) has been used extensively in recent years to assess both close and distant relationships among many kinds of organisms as a result of the existence of conserved sequences within this region, and the concerted evolution among units (Kurtzman and Robnett 1998). Using the information from this region, different methods have been developed for yeast species identification.

3.1.1 Ribosomal RNA Gene Sequencing

The determination and comparison of nucleotide sequences for the purpose of recognizing similarities or differences between organisms is at the heart of any molecular approach. Therefore, choosing the appropriate gene is paramount to the success or failure of any such sequence analysis between species. The 26S, 18S, 5.8S, and 5S ribosomal genes have undergone a relatively slow evolution, allowing for their use in the comparison of distantly related organisms. Work has

Table 1 Main enzymatic activities described in non-*Saccharomyces* wine yeasts

Enzymatic activity	Yeasts	References
Protease	<i>Candida</i> , <i>Kloeckera</i> , <i>Pichia</i>	Charoenchai et al. 1997
β-Glucosidase	<i>Candida</i> , <i>Debaryomyces</i> , <i>Hanseniaspora</i> , <i>Hansenula</i> , <i>Kloeckera</i> , <i>Kluyveromyces</i> , <i>Metschnikowia</i> , <i>Pichia</i> , <i>Saccharomyces</i> , <i>Schizosaccharomyces</i> , <i>Zygosaccharomyces</i>	Grossmann, et al. 1987; Rosi et al. 1994; Manzanares et al. 2000
Esterase	<i>Brettanomyces</i> , <i>Debaryomyces</i> , <i>Rhodotorula</i>	Besançon et al. 1995
Pectinase	<i>Candida</i> , <i>Cryptococcus</i> , <i>Kluyveromyces</i> , <i>Rhodotorula</i>	McKay 1990
Lipase	<i>Candida</i>	Charoenchai et al. 1997

Table 2 Studies about wine yeast identification using molecular biology techniques

Methodology	Genus	References
AFLP	<i>Saccharomyces</i>	de Barros Lopes et al. (1999)
δ elements	<i>Saccharomyces</i>	Ness et al. (1993)
Intron splice site	<i>Saccharomyces</i>	de Barros Lopes et al. (1999)
Karyotype	<i>Saccharomyces</i>	Cardinali and Martini, 1994; Guillamón et al. (1996); Ibeas et al. (1997); Martínez et al. (1995)
	<i>Hanseniaspora</i>	Schütz and Gafner (1994)
	<i>Zygosaccharomyces</i>	Török et al. (1993)
Microsatellite	<i>Saccharomyces</i>	Baleiras Couto et al. (1996)
Nested-PCR	<i>Brettanomyces</i>	Ibeas et al. (1996)
Plasmids	<i>Saccharomyces</i>	Pearson and McKee (1992)
	<i>Zygosaccharomyces</i>	Pearson and McKee (1992)
RAPDs	<i>Saccharomyces</i>	Baleiras Couto, (1996); Quesada and Cenis (1995)
	<i>Metschnikowia</i>	Lopandic et al. (1996)
	<i>Rhodotorula</i>	Quesada and Cenis (1995)
	<i>Zygosaccharomyces</i>	Baleiras Couto et al. (1994)
	<i>Candida</i>	Quesada and Cenis (1995)
	<i>Picchia</i>	Quesada and Cenis (1995)
	<i>Torulaspora</i>	Quesada and Cenis (1995)
	<i>Hansenula</i>	Quesada and Cenis (1995)
RFLP-karyotype	<i>Candida</i>	Versaud and Hallet (1995)
	<i>Kloeckera</i>	Versaud and Hallet (1995)
	<i>Schizosaccharomyces</i>	Versaud and Hallet (1995)
RFLP-mtDNA	<i>Saccharomyces</i>	Constantí et al. (1997); Guillamón et al. (1996); Ibeas et al. (1997); Martínez et al. (1995); Querol et al. (1992a); Querol et al. (1994)
	<i>Kluyveromyces</i>	Belloch et al. (1997)
	<i>Zygosaccharomyces</i>	Guillamón et al. (1997)
	<i>Brettanomyces</i>	Ibeas et al. (1996)
RFLP-ITS/5.8S rRNA gene	<i>Candida</i>	Constantí et al. (1997); Guillamón et al. (1998)
	<i>Hanseniaspora</i>	Guillamón et al. (1998)
	<i>Saccharomyces</i>	Guillamón et al. (1998)
	25 different genera	Esteve-Zarzoso et al. (1999)

focussed primarily on the D1/D2 domain of the 26S rDNA (Kurtzman and Robnett 1998) and on the 18S subunit (James et al. 1997). The techniques have gained substantially from the introduction of the polymerase chain reaction, direct sequencing, and the availability of numerous nucleotide databases, which contain sequence information on a diverse range of organisms.

3.1.2 Restriction Fragment Length Polymorphism of RDNA

Recently, Esteve-Zarzoso et al. (1999) proposed a rapid and easy method for routine yeast identification, based on RFLPs of the 5.8S rRNA gene and the internal transcribed spacers (ITS1 and 2). They presented an initial database for the identification of more than 132 yeast species belonging to 25 genera, most isolated from food. This is the first available molecular method that provides information to identify a large number of yeast species in an easy and quick way. This

database has been improved to identify 300 yeast species (<http://motor.edinfo.es/iata>). Using this method, it is possible to identify yeasts from isolated colonies or directly from food samples (see Figure 1). In addition, the anamorph and teleomorph forms yielded the same patterns and all the strains of the same species also exhibit the same pattern. Guillamón et al. (1998) used this method to identify 33 wine yeast species and Fernández-Espinar et al. (2000) identified all the species of the genus *Saccharomyces*, including the flor yeast responsible for biological ageing in the process of making “fino” sherry wine. The flor *S. cerevisiae* strains exhibited restriction patterns different from those typical of the species *S. cerevisiae*, due to the presence of a 24-bp deletion located in the ITS1 region. Esteve-Zarzoso and Peris-Torán (2001) detected this specific “flor” pattern in all the strains isolated from the velum of sherry wines from wineries located in Jerez, Spain.

Using the same methodology, but amplifying a different region (18S rRNA and ITS1) Dlačhy et al. (1999) also

constructed a database of restriction fragment patterns to identify 128 species associated mainly with food, and fermented drinks.

3.2 Methods for Yeast Characterization

Methods by which strains of the same species can be differentiated have been shown to be very important for yeast strain characterization. In winemaking, several studies have analyzed the diverse microflora of grapes and musts and several interesting methods have been developed (Figure 2).

PCR amplification using intron splicing site (consensus sequences that flank introns) displays polymorphism mainly at the within-species level (de Barros Lopes et al. 1996); PCR amplification of delta elements (repeat sequences that flank the TY1 retrotransposons) has been widely used to characterize wine yeast strains of *Saccharomyces cerevisiae* (Ness et al. 1993). Another PCR-based approach, known as random amplified polymorphic DNA (RAPD), consists of the amplification of random segments of DNA with a single and short (from 5- to 15-mer) primer of an arbitrary nucleotide sequence. The level of differentiation, either interspecific or intraspecific, depends highly on the primer used. However, one of the most important problems with this technique is the low stable (nonrepetitive) patterns obtained.

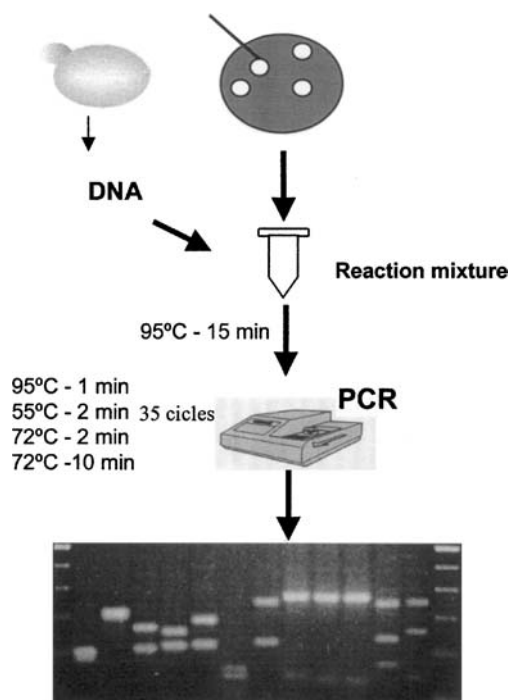


Figure 1 Scheme of the yeast identification using Restriction fragment length polymorphism of the 5.8S rRNA and the internal transcribed spacers (Esteve-Zarzoso et al. 1999).

The amplified fragment length polymorphism (AFLP) was used to investigate genetic variation in commercial strains and winery isolates (de Barros Lopes et al. 1999). It was proposed that AFLP is a very useful method for discriminating yeasts at both species and subspecies levels, and also to characterize hybrids (de Barros Lopes et al. 2002). Although initially more labor intensive than other PCR techniques, the reproducibility of the results is the advantage over the previous PCR method described.

The analysis of mitochondrial DNA restriction fragment length polymorphisms (mtDNA-RFLP) has long been used as a method for characterizing wine yeast strains (Vezinhet et al. 1990). However, due to the complexity associated with the procedure required to isolate a sufficient amount of mtDNA, its use for routine analysis was limited until quite recently. The last decade has seen improvements in the process (Querol et al. 1992b), removing the need for specialized equipment and reducing the complexity and time scale, while retaining the discriminatory power and reproducibility (Figure 3). This simplified technique has been used successfully to characterize yeast strains of species belonging to genera *Brettanomyces*, *Candida*, *Debaryomyces*, *Kluyveromyces*, *Saccharomyces*, and *Zygosaccharomyces* (see Table 2).

Chromosomal DNA profile analysis (electrophoresis karyotyping). The analysis of chromosomal DNA polymorphisms due to chromosome rearrangements has proved to be useful for the differentiation of species (and also strains) belonging to several genera, e.g., *Candida*, *Kluyveromyces*, *Saccharomyces*, and *Zygosaccharomyces*. In the case of wine yeast, it has been used for yeast characterization and was applied to analyse the dynamics of the yeast populations (Querol and Ramón 1996) and in the characterization of the industrial wine yeast (Fernández-Espinar et al. 2001).

In the following section we will discuss the resolution of some of these techniques and their industrial application for the study of yeast population dynamics during natural and inoculated wine fermentations and for the characterization of *S. cerevisiae* strains of industrial interest.

3.3 Study of Yeast Population Dynamics During Natural Wine Fermentations

Using mtDNA RFLP Querol et al. (1994) analyzed the population dynamics of *Saccharomyces* strains during spontaneous wine fermentations. They found that despite the high diversity of strains observed at the beginning of the fermentation, very few dominated the process. However, when similarities in the mtDNA restriction pattern of the different *S. cerevisiae* strains (measured as the proportion of shared restriction fragments) were low, a clear sequential substitution of predominant strains was observed during each fermentation phase. When the similarity was high, even though a sequential substitution could also be observed between secondary strains, a clearly predominant strain was present during the whole fermentation process.

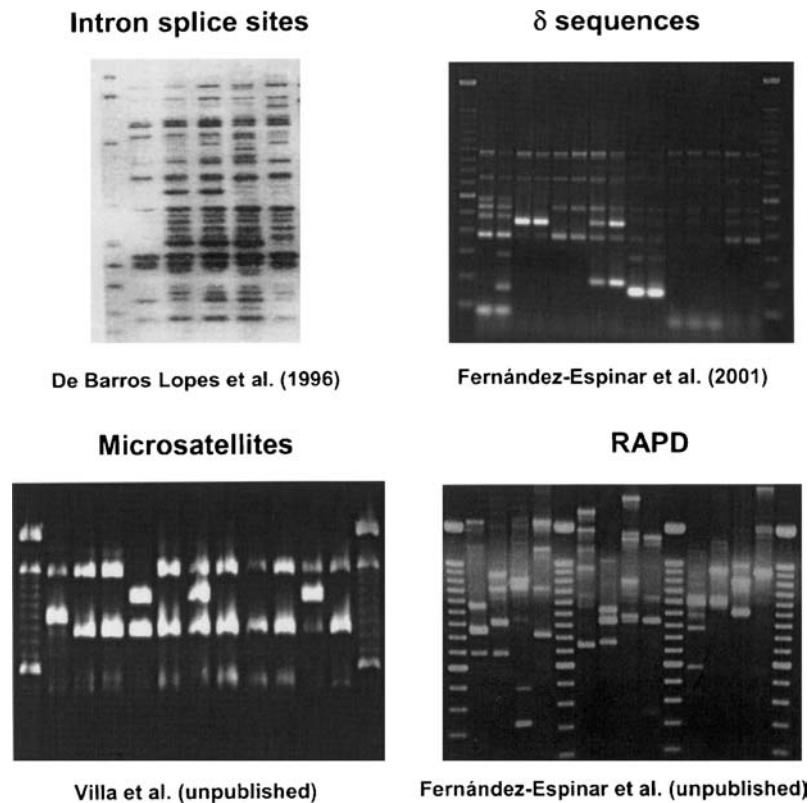


Figure 2 Molecular methods to yeast characterization: intron splice sites, δ sequences, microsatellites and RAPD.

On the basis of chromosomal profiles, Frezier and Dubourdieu (1992) found that a small number of *S. cerevisiae* strains were capable of dominating fermentations in the same winery over two vintages, independently of the grapevine cultivar. Vezinhet et al. (1992) investigated using mtDNA analysis and electrophoretic karyotyping the evolution of *S. cerevisiae* strains isolated during six consecutive years in the cellars of two vineyards. They concluded that the wide distribution of some strains in the

areas studied and their presence over a number of years constituted evidence for the occurrence of specific native strains representative of a particular wine region. Schütz and Gafner (1994) proposed that the composition of yeast strain populations differs from must to must and from year to year. However, they also found strains isolated from two grape musts and from two vintages that presented very similar chromosome patterns to the commercial strain W27 (Lallemand) isolated from the same region.

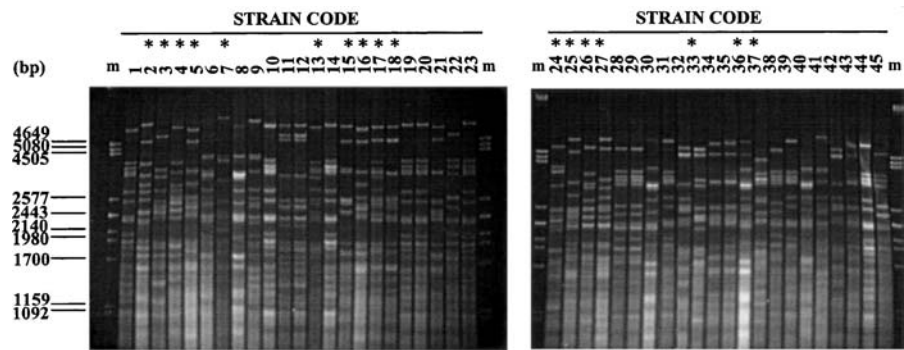


Figure 3 *HinfI* mtDNA restriction patterns of commercial wine yeast strains analyzed in Fernández-Espinar et al. (2001).

Guillamón et al. (1996) have analyzed the correlation between genetic distance and ecological/geographical factors using mtDNA restriction analysis and electrophoretic karyotyping. This constitutes the first survey to investigate molecular variation among natural strains from different wine regions. They found significant correlation between both ecological factors (e.g., grape varieties) and/or geographical origin and the molecular relationships among strains. The analysis of genetic variation in natural yeast populations often results in the determination of the particular biological factors that influence population structure, hence population variations within and between wine regions have to be characterized to elucidate those factors that determine the distribution of variations in natural populations of *S. cerevisiae*.

3.4 Study of Yeast Population Dynamics During Inoculated Wine Fermentations

Using mtDNA restriction patterns, Querol et al. (1992a) conducted the first study of population dynamics and the roles played by the active dry yeast strain and the natural *Saccharomyces* flora during inoculated industrial fermentations. It was demonstrated that the inoculated strains T73 (Lallemand) compete with the natural strains but do not completely suppress their growth until three to six days after inoculation. However, the predominance of the inoculated strain was evident at the end of the fermentations.

Using physiological tests and karyotyping, Schütz and Gafner (1993) analyzed the succession of three different dried yeasts at three different time points of wine fermentation. *Hanseniaspora uvarum* was present at the beginning of the fermentation, whereas only the inoculated strains of *S. cerevisiae* were observed in the middle and at the end of the fermentation. However, as a consequence of the few strains, these authors did not detect as much diversity as that observed by Querol et al. (1992a).

The use of active dry yeasts is of particular interest for the wine industry. It is generally assumed, as we discussed previously, that indigenous yeasts are suppressed by the starter. However, some studies showed that native strains are better adapted to fermentation conditions than commercial strains (Esteve-Zarzoso et al. 2000; Esteve-Zarzoso and Peris-Torán 2001).

3.5 Characterization of Commercial Wine Yeast Strains

Since numerous yeast strains are being produced for the commercial wine market, it is interesting to study the degree of relatedness between commercial *Saccharomyces* strains. Fernández-Espinar et al. (2001) characterized 45 selected yeasts from 10 different yeast producers. Three molecular techniques, namely mtDNA restriction analysis (Figure 3),

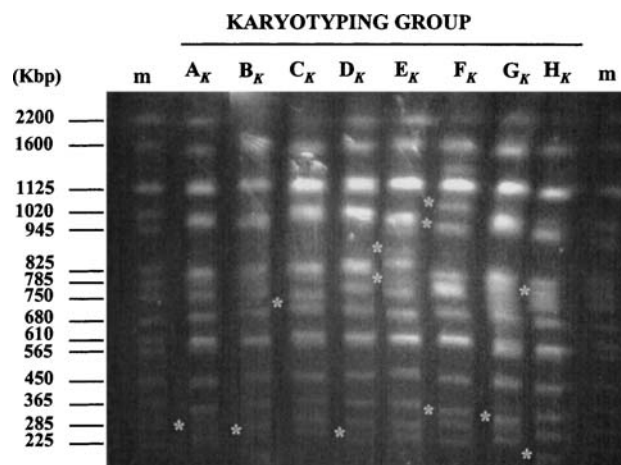


Figure 4 Chromosomal profiles of commercial wine yeast strains analyzed in Fernández-Espinar et al. (2001).

electrophoretic karyotyping (Figure 4) and specific PCR of δ sequences (Figure 2), were used for this purpose. The maximum discriminatory power was obtained when the results of the three techniques were combined. The results showed evidence of mistakes during production or fraudulent practices by yeast producers, since the same strains commercialized under different names or the same name is used for different products by different companies.

3.6 Future Methods

The use of PCR in molecular microbiology has increased to the point where it is now accepted as the golden standard for detecting nucleic acids from a number of origins and has become an essential tool in the research laboratory. Real-time PCR has engendered wider acceptance of PCR, due to its improved rapidity, sensitivity, reproducibility, and the reduced risk of carry-over contamination (Mackay et al. 2002). Traditional detection of amplified DNA relies upon electrophoresis of the nucleic acids in the presence of ethidium bromide and visual observation after irradiation by ultraviolet light. Southern blot detection of the amplicon using hybridization with a labeled oligonucleotide probe is also time consuming. The detection of the amplicon and the possibility of visualizing it as the amplification progresses is the foundation of real-time PCR. The monitoring of the accumulation of the amplicon in real time has been made possible by the labeling of primers with fluorogenic molecules. Due to the numerous advantages, these methods probably are an alternative to the techniques described previously, not only for the detection and identification of spoilage yeast or for wine yeast monitoring during the alcoholic fermentation. Thus, this technology has also been applied in studies of yeast gene expression during the alcoholic fermentation.

4 STRUCTURAL AND FUNCTIONAL WINE YEAST GENOMICS

4.1 Genomic Characteristics

Saccharomyces cerevisiae cells are generally ellipsoidal in shape, the vegetative reproduction is by multilateral budding and its vegetative phase is predominantly diploid, the only haploid stage is the ascospore. *S. cerevisiae* has a relatively small genome, a large number of chromosomes, little repetitive DNA, and few introns. Haploid strains contain approximately 12–13 megabases of nuclear DNA, distributed along 16 linear chromosomes whose sizes vary from 250 to 2000 kb (Barre et al. 1992; Pretorius 2000). Most *S. cerevisiae* strains used in the laboratory are either haploid or diploid and have a defined set of chromosome lengths. However, wine strains are mainly diploid, aneuploid, or polyploid (Codón and Benítez 1995). Aneuploidy and polyploidy may confer advantages to adapt to variable external environments or, perhaps, is a way to increase the dosage of some genes important for fermentation (Bakalinsky and Snow 1990; Salmon 1997). The meiotic segregants from wine strains diploidise with high frequency, indicating a high frequency of homothallism. Heterozygosity has been observed in both homothallic and heterothallic strains (Barre et al. 1992; Codón and Benítez 1995).

Wine yeasts also show a high level of chromosome length polymorphism (Bidenne et al. 1992; Rachidi et al. 1999). This polymorphism is generated mainly by illegitimate recombination mediated by Ty transposons or subtelomeric repeated sequences. This feature has several practical consequences: sporulation ability is very variable, between 0 and 75% ascus formation can be observed on a sporulation medium and spore viability is also highly variable, ranging from 0 to 98% (Codón and Benítez 1995; Mortimer et al. 1994).

4.2 Adaptive Evolution of Wine Yeast Strains

Although the origin of *S. cerevisiae* is a matter of controversy, its original genome has been subjected to strong selective pressures since its first unconscious use in controlled fermentation processes. Useful phenotypic traits, such as fast growth in sugar-rich media, high alcohol production and tolerance, and good flavor production, which have been selected over billions of generations, have had strong influences on the *S. cerevisiae* genome (Pérez-Ortín et al. 2002). We now will analyze some of the molecular mechanisms that explain this wine yeast adaptation to vinification.

4.2.1 Stress Adaptation

During the alcoholic fermentation, yeast cells are subject to a number of stresses (Attfield 1997), the most important being osmotic and ethanol stresses. Osmotic stress is due to the high sugar content in the must and yeast cells must resist this stress

in order to start growing and to carry out the alcoholic fermentation. Ethanol stress is related to the progressive production of this compound throughout vinification. Alcohol is highly toxic to yeast metabolism and growth (Ingram and Buttke 1984) and the cell membrane is the primary target for its action. Also it is important to mention the stress due to glucose starvation, which could take place towards the end of the fermentation stage. Supraoptimal temperatures constitute another kind of stress conditions that can take place during fermentation, although it is not very significant for most vinifications, given the sophisticated temperature control systems currently used in wineries (Fleet and Heard 1992). Similarly, adverse growth conditions, such as high ethanol and acetaldehyde concentrations and oxidative stress due to respiratory metabolism, can be found in the biological ageing of fino sherry wine. Acetaldehyde is a known inhibitor of a wide range of metabolic activities and is more toxic than ethanol (Jones 1990). Response to stress conditions requires the activation of signal transduction pathways, which involves the synthesis of protective molecules, including heat shock proteins. One of these proteins (Hsp104p) has been shown to be responsible for stress tolerance in laboratory yeast strains carrying out respiratory metabolism (Lindquist and Kim 1996), but not for wine yeast strains growing under fermentation conditions in a glucose-rich medium (Carrasco et al. 2001) or for brewery yeast strains (Brosnan et al. 2000). Aranda et al. (2002) analyzed the responses of several *S. cerevisiae* strains (some of them isolated during the production of fino sherry wine) to several adverse conditions. In strains that are dominant during biological ageing, there was a clear correlation between resistance to ethanol and acetaldehyde, the high induction of *HSP* genes by these compounds and its presence as the predominant strain in most levels of several “soleras.” It is interesting to note that the dominant strains during biological ageing are the less resistant ones and the strains isolated during the alcoholic fermentation are more resistant to the osmotic stress.

4.2.2 Gene Expression Variability in Wine Yeast

The molecular basis of the technological properties of wine yeast strains are still largely unknown. However, the obvious possibility is that the adaptation of these strains to the enological environment is dependent on specific expression profiles of their genomes (for a review see Pérez-Ortín et al. 2002). Comparative analyses of gene expression between industrial and nonindustrial strains and between different industrial strains could lead to the identification of genes involved in the fitness of the strains in industrial environments. To date, the study of gene expression during wine fermentation has focused on genes induced in the stationary phase in order to express specific activities at the end of the process (Puig et al. 1996; Puig et al. 2000; Riou et al. 1997).

Due to the different properties of the particular wine strains, it seems of great interest to study real commercial wine strains. Consequently, Puig et al. (1996); Puig and

Pérez-Ortín (2000) determined the levels and expression patterns of several genes during wine microvinifications by using a commercial strain (T73, Lallemant). Genes such as *POT1*, *HSP104*, and *SSA3*, which are expressed late in laboratory culture conditions, are expressed in wine yeast cells only during the first few days in microvinifications. The reason for this could be the very different growth conditions used in microvinification (and therefore in real vinifications) compared to those of laboratory conditions. Vinifications are characterized by very high contents of glucose and fructose (20–25%) compared with the usual laboratory conditions (1–3%), and anaerobic conditions vs highly aerated laboratory conditions (Puig et al. 1996).

The completion of the sequence of the genome of *S. cerevisiae* allowed the development of tools for the evaluation of the expression of the entire complement of genes encoded in the genome (transcriptome). The DNA microarray hybridization analysis was used to investigate how interesting genes change their expression during a biological process; several attempts have been made with wine yeast (Cavalieri et al. 2000; Backhus et al. 2001; Hauser et al. 2001). The knowledge of genetic features as well as the specific expression profiles wine yeast strains under different growth conditions could help us to understand better the biological process of fermentation at the molecular level and how the gene expression is regulated in relation to changes in the physical and chemical properties of the growth medium.

It is surprising that the genes involved in sulphur (*SUL1-2*) and ammonia (*MEP2*) transport (Cavalieri et al. 2000), or that (sulphite efflux, *SSU1*) involved in sulfite resistance (Hauser et al. 2001), were found to be overexpressed in wine yeast strains. They investigated in great detail the possible mechanisms for regulation of the expression of the *SSU1* gene of the T73 wine yeast strain. A rearrangement of the promoter of *SSU1* was detected, leading to up-regulation in its expression. This can be interpreted as being fixed through its evolution as a result of the selective pressures imposed by winemaking procedures (Pérez-Ortín et al. 2002).

4.2.3 Wine Yeast Genome Evolution

The flexibility of the yeast genome to adapt to externally introduced environmental changes has been demonstrated in several experiments. Thus, it has postulated that an ancient duplication of the entire yeast genome may have been instrumental in its evolutionary adaptation to anaerobic, fermentative growth by providing new and specialized gene functions (Wolfe and Shields 1997). Wine yeast strains have the ability to reorganize their chromosomes during mitotic growth (Longo and Vezinhet 1993). In contrast to laboratory strains, wine yeast strains seem not to remain genetically uniform because of this exacerbated capacity to undergo genomic changes (Pretorius 2000). A reason for this is the ability of many “natural” strains to change their mating type when haploid (homothallism). Using this capability, yeast cells could evolve very quickly by means of three successive steps: sporulation (producing haploid cells thorough meiotic

reduction), mating type switching of the daughter cells and conjugation with any of the mothers of the same single-spore colony (Mortimer et al. 1994). This process (called “genome renewal”) would produce highly homozygous strains that eliminate deleterious mutations by natural selection. Yeast strains, however, are usually aneuploid (Bakalinsky and Snow 1990; Guijo et al. 1997) and heterozygous for many loci (Barre et al. 1992; Kunkee and Bisson 2000), which is not in agreement with the genome renewal hypothesis (Puig et al. 2000). There are several other ways in which wine strains change over time. Apart from spontaneous mutation, which occurs at comparatively very low rates, Ty-promoted chromosomal translocations (Rachidi et al. 1999), mitotic crossing-over (Seehaus et al. 1985) and gene conversion will provoke faster adaptation to environmental changes (Puig et al. 2000). Extra copies of chromosome XIII, which contains the alcohol dehydrogenase genes *ADH2* and *ADH3*, are found in flor wine yeasts, these genes are of special interest to oxidize ethanol (Guijo et al. 1997). Finally, (Pérez-Ortín et al. 2002) found that the *SSU1-R* allele, which confers sulfite resistance to yeast cells, is the product of a reciprocal translocation between chromosomes VIII and XVI due to unequal crossing-over mediated by microhomology between very short sequences on the 5' upstream regions of the *SSU1* and *ECM34* genes, which put the *SSU1* coding region under the control of the *ECM34* promoter. They also showed that this translocation is only present in wine yeast strains, suggesting that the use of sulfite as a preservative in wine production over millennia could have favored its selection. This is the first time that a gross chromosomal rearrangement has been shown to be involved in the adaptive evolution of *S. cerevisiae*.

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Yeast in the Dairy Industry

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1 INTRODUCTION

Yeast have been isolated from all kinds of dairy products (Fleet 1990; Fleet and Mian 1987; Jakobsen and Narvhus 1996; Rohm et al. 1992; Tilbury et al. 1974). Yeast are used as important starter cultures in food products such as bread, beer, wine, traditional fermented foods (Fleet 1990; Fleet and Mian 1987; Jakobsen and Narvhus 1996) and seems to be the most important micro-organism exploited by man. The role of yeast as micro-organisms, which could have a positive influence on the quality of dairy products, especially cheese, has not been widely accepted (Fleet 1990; Jakobsen and Narvhus 1996). Instead, yeasts have often been seen as spoilage organisms originating from poor hygiene and sanitation (Fleet and Mian 1987; Jakobsen and Narvhus 1996). The nutritional requirement of yeast, the ability to grow at low temperature, low pH, low moisture content, and high salt or sugar concentrations together with their enzymatic activity make yeast a natural part of the microbiota in dairy products. In 1995 the first symposium on “Yeasts in the dairy industry: Positive and Negative Aspects” was organized and held by the International Dairy Federation (IDF) expert group F47—Yeast. As reflected in the published literature the interest in yeast in the dairy industry has increased since and the knowledge of the positive and negative role played by yeast in dairy products has become more detailed. The key areas have been: (a) study of important technological characteristics of yeast, e.g., proteolytic and lipolytic activity, aroma formation and fermentation and/or assimilation of residual sugars and lactate and citrate and pigment formation especially related to different kind of cheese; (b) properties affecting both the negative role of yeast as spoilage organism causing quality defects as well as the potential of yeast as highly controlled

starter cultures; (c) yeast classification and taxonomy based upon molecular methods. The present review will deal with the positive and negative aspects of yeast in the dairy industry.

A variety of yeast species has been isolated from milk and dairy products (Fleet 1990; Fleet and Mian 1987; Gadaga et al. 2001; Rohm et al. 1992; Roostita and Fleet 1996a,b; van den Tempel and Jakobsen 1998; Tilbury et al. 1974; Tudor and Board 1993; Westall 1998) but a few main species are frequently detected in dairy products. These yeast are representatives of the genera *Debaryomyces*, *Kluyveromyces*, *Yarrowia*, *Candida*, and *Saccharomyces* (Viljoen 2001). The yeast occur both in the teleomorphic and the anamorphic state in dairy products, depending on the yeasts' ability to produce ascospores. Some of the most common yeast related to dairy products mentioned first by their teleomorphic then by their anamorphic state are: *Debaryomyces hansenii*/*Candida famata*, *Galactomyces geotrichum*/*Geotrichum candidum*, *Yarrowia lipolytic*/*Candida lipolytica*, *Kluyveromyces marxianus*/*Candida Kefyr*, *Kluyveromyces lactis* var. *lactis*/*Candida spherical*, and *Saccharomyces cerevisiae*/*Candida robusta*.

To describe yeast occurring in dairy products and select and maintain yeast as a starter culture, identification at subspecies level is a necessity. The taxonomy of yeast is mainly based on the criteria described in the third and fourth edition of the taxonomical study “The Yeasts” edited by Kreger van Rij (1984) and Kurtzman and Fell (1998), respectively. The criteria includes micro- and macro-morphology in standard media, assimilation, and fermentation profiles of standard carbohydrates, growth at different temperatures and under defined osmotic pressure, development of pseudo or true mycelium, formation of ascospores as well as other phenotypic criteria. It takes time and experience

to conduct a correct classical identification. Therefore, easier identification systems like API-ZYM systems (Biomerieux, Macy l'Etoile, France) are widely used. The API-test is based on assimilation profiles of the yeast. The API-kit's were originally made to offer a fast identification of yeast of medical importance. Several investigations have found food-related yeasts that were not included in the reading key of the kit (Deák and Beuchat 1988). The enlarged version of the test, API-kit 32C, has been found to be reliable in assisting in yeast identification and acceptable for a rapid identification of yeasts associated to cheese, as long as it is used in combination with the classical methods, especially when a reliable determination of the sexual form is required, for the identification (van den Tempel 2000). Other methods based on criteria such as profiles of volatile metabolites of the yeast (Magan et al. 2001; Westall 1998), analysis of volatile profiles of yeast by an electronic nose (Magan et al. 2001) and identification based on Fourier-transform of the specific infrared spectrum of the biochemical compounds of the dried yeast cell (Kümmerle et al. 1998) have been reported as methods for fast identification at species level.

The number of yeast genera and yeast species has increased 100% or more since 1970 where the first edition of "The Yeasts" was published. At the same time yeast species described as belonging to different genera or species have been proven to belong to the same species. This shows the complexity of the yeast taxonomy but also the growing interest in yeast classification. One of the major reasons for the changes in yeast classification and taxonomy is the use of molecular methods including analyses of whole chromosomes and as with other micro-organisms, the classification of yeast is based more and more on genotypic rather than phenotypic criteria for yeast isolated from dairy products. Several methods have been reported for taxonomical use, for e.g., molecular techniques using SDS-gelelectrophoresis of whole-cell protein pattern, pulse field gelelectrophoresis (PFGE) (Montrocher et al. 1998; Esteve-Zarzoso et al. 1999; Gente et al. 2002; Jespersen and Kühle 2000), restriction fragment length polymorphism analysis of mitochondrial DNA (DNA-RFLP) (Petersen et al. 2002; Romano et al. 1996; van den Tempel and Jakobsen 2000), internal genomic spacer sequences (IGS), 18S rRNA analysis (Cappa and Cocconcelli 2001) and polymerase chain reaction—restriction fragment length polymorphism (PCR-RFLP) analysis of the intergenic transcribed spacer region (ITS) (Caggia et al. 2001; Petersen et al. 2001). However, for many of the yeast genera related to dairy products the molecular methods are not yet fully developed or sensitive enough to separate strains on species or subspecies level.

2 PROTEOLYTIC ACTIVITY

Published results on the proteolytic activity of yeast isolated from dairy products are mainly based upon the ability to produce a clearing zone in casein and skim milk agar. It has been demonstrated (Hansen and Jakobsen 2001; Larsen et al.

1998) that the agar diffusion tests may not offer the sensitivities required to detect proteolytic activity. Methods such as gelelectrophoresis, high performance liquid chromatography (HPLC), or capillary electrophoresis (CE) give a different and more detailed picture, which also demonstrates that the proteolytic activity of yeast is strain specific (Hansen and Jakobsen 2001; van den Tempel 2000). Several studies of strains of *D. hansenii* have indicated that the examined strains could not degrade casein (Hansen and Jakobsen 1997). However, low proteolytic activity was observed in *D. hansenii* isolated from Picante cheese (Freitas et al. 1999) and blue veined cheese (van den Tempel 2000). Similar variability in the proteolytic activity for strains of *K. lactis* has been observed (Grieve et al. 1983; Hansen and Jakobsen 1997; Roostita and Fleet 1996a). However, Grieve et al. (1983) did show that a strain of *K. lactis* was able to hydrolyze α -, β -, and κ -casein, which indicates that some strains are able to contribute to the maturation of cheese.

Gueguen and Lenoir (1975) examined 30 strains of *G. geotrichum* and found that they produced extracellular and intracellular proteinases and peptidases. Extracellular activity was present in 66% of the strains, which could be divided into two groups of 25% showing high proteolytic activity and 75% having low proteolytic activity. Optimum pH for the proteolytic activity was close to 5.5. Other investigations of commercial strains of *G. geotrichum* used in cheese production showed that the proteolytic activity was weak when present.

Yarrowia lipolytica produces several proteinases and many of these are extracellular. The secretion of both alkaline and acid proteinases has been detected (Ogrydziak et al. 1982; Vasileva-Tonkova et al. 1996). The activity of the extracellular acid proteinase at pH 5.2 was optimal at 15°C but was significantly reduced at 8°C. Many investigations of *Y. lipolytica* have shown that different strains of *Y. lipolytica* have proteolytic activity and are able to breakdown all the casein components. (Freitas et al. 1999; van den Tempel and Jakobsen 2000; Wyder and Puhon 1999) and seems to be at the same level as a strong proteolytic strains of *P. roqueforti* (van den Tempel 2000).

Candida catenulata seems to have a high proteolytic activity similar to the level of *Y. lipolytica* (Roostita and Fleet 1996a), but detailed studies have not been reported.

Saccharomyces cerevisiae can be proteolytic but the majority of strains do not show proteolytic activity (Hansen and Jakobsen 1997; Hansen and Jakobsen 2001; Roostita and Fleet 1996a). The proteolytic activity was studied by Hansen and Jakobsen (2001) using CE for five selected *Saccharomyces* spp. being a commercial starter cultures in blue veined cheese, an isolates from blue veined cheeses, a type strains of *Saccharomyces* spp. and two starter cultures of *Saccharomyces* spp. used in other food fermentation. The CE results for the five strains showed that only the commercial blue cheese strain of *S. cerevisiae* could break down casein. The CE-profiles showed that *S. cerevisiae* was able to hydrolyze some of the α_{s1} - and β -casein. Differences in the CE-profiles indicated a synergistic effect in the degradation of casein by

S. cerevisiae and *Penicillium roqueforti*, seen as higher number and different grouping of peptides when grown together compared to growth of the individual culture.

3 LIPOLYTIC ACTIVITY

Almost all yeast related to cheese have esterase activity and are able to hydrolyze tributyrin (Fleet and Mian 1987; Hansen and Jakobsen 1997; Roostita and Fleet 1996a; van den Tempel and Jakobsen 1998). This applies for *D. hansenii*, *K. lactis*, *S. cerevisiae* and *Y. lipolytica*, *C. catenulata* and *C. geotrichum*, all showing esterase activity.

The reported results on the esterase activity of *D. hansenii*, *K. lactis*, and *S. cerevisiae* are contradicting (Besancon et al. 1992; Fleet and Mian 1987; Hansen and Jakobsen 1997; Roostita and Fleet 1996a; van den Tempel and Jakobsen 1998). But in general the activity is low. Tested in API-ZYM systems (Biomerieux, Macy l'Etoile, France), *S. cerevisiae*, *D. hansenii*, and *K. lactis* could hydrolyze the fatty acid with chain length of C:8, but not the fatty acid with a chain length of C:14 (Hansen and Jakobsen 2001). Lipolysis of long-chain fatty acids has been demonstrated for *Y. lipolytica*, *C. catenulata*, and *C. geotrichum* and the activity seems to be at the same level for the three yeasts (Roostita and Fleet 1996b; van den Tempel 2000).

Isolation of several intra and extracellular lipases from *Y. lipolytica* has been reported and the strong lipolytic activity of *Y. lipolytica* seems mainly to be due to extracellular lipases with optimal activity at pH 6–10 (Destain et al. 1997; Pereira-Meirelles and Rocha-Leão 1997). Some of the reported lipases are quite stable and are still active after 370 days at 5°C (Pereira-Meirelles and Rocha-Leão 1997). To demonstrate the extent of the lipolytic activity of *Y. lipolytica*, five strains were compared with five different strains of *P. roqueforti* with medium to high lipolytic activity. It was found that the lipolytic activity was twice the activity of the strongest lipolytic strain of *P. roqueforti* (van den Tempel and Jakobsen 2000). Investigations comparing lipolytic activity after growth under different condition showed that the lipolytic activity was depending on pH, temperature, and the growth media (Hansen and Jakobsen 1997; van den Tempel 2000).

4 YEAST IN DAIRY PRODUCTS

4.1 Farm Milk

Often the level of yeast in raw milk is found to be 10^2 – 10^4 cells/ml (Lagneau et al. 1996; van den Tempel and Jakobsen 1998). The dominant yeast species detected in raw milk in Denmark, Italy, and Belgium were found to be *C. famata*, *C. lipolytica*, and *Trichosporon cutanum* (Corbo et al. 2001; Lagneau et al. 1996; van den Tempel and Jakobsen 1998). The study of raw milk in Belgium indicated that yeast count was depending on whether the milk came

from cows with or without mastitis. Yeast were detected in more than 50% of samples from quarters with mastitis infections, but only in 25% of the samples from the noninfected quarters (Lagneau et al. 1996). Yeast have been detected in the number of 10^3 cfu/ml in pasteurized milk (Fleet and Mian 1987; Lagneau et al. 1996; van den Tempel and Jakobsen 1998) and most of the common species in raw milk were able to grow to 10^8 or 10^9 cfu/ml in UHT milk even at low temperatures (Fleet and Mian 1987; Roostita and Fleet 1996b). Spoilage of raw milk and pasteurized milk by yeast are not reported as a very common problem. The occurrence of yeast in raw milk is assumed to be a contamination source of other dairy products. Reported investigations using molecular techniques to follow yeast at subspecies level through the whole production and to trace the origin of the yeast are limited.

In sweetened condensed milk the low water activity, due to high concentrations of sugar favor yeast as a potential spoilage organism while bacterial growth is inhibited. Yeasty taste and flavor and gas production are known spoilage by yeast in this product (Walker and Ayers 1970).

4.2 Cream and Butter

Yeast have been reported to cause spoilage such as yeasty flavor and foamy appearance in all kind of cream products (Fleet and Mian 1987; Walker and Ayers 1970). In an Australian study 48% of the examined cream samples contained yeast in the range of 10^3 – 10^4 cfu/ml while a number of 10^4 – 10^5 were found in 14% of the samples (Fleet and Mian 1987). The yeast identified in the study were mainly *C. famata*, *Rhodotorula glutinis*, *C. diffluens*, *Cryptococcus laurentii*, and *R. rubra*. These yeast species all showed high lipolytic activity and were able to grow at 5°C.

An investigation from Italy showed yeast in 4 out of 5 butter samples examined (Minervini et al. 2001). The detected yeast included *C. kefir*, *C. guilliermondii*, and *S. cerevisiae* in concentrations of 10^3 – 10^5 cfu/g (Minervini et al. 2001). In 1987 Fleet and Mian found yeast in 7 out of 16 butter samples. The yeast occurred in numbers below 10^3 per gram and belonged mainly to the species *R. glutinis* and *Rhodotorula rubra*. Lipolytic yeast on the butter surface has also been reported (Walker and Ayers 1970), but all together the literature on this subject is very limited.

4.3 Yoghurt

Yoghurt is an acidic fermented milk product, which normally has acetaldehyde in the final aroma. It is made of milk inoculated with thermophilic lactic acid starter cultures. During fermentation about 35% of the lactose is hydrolyzed to glucose and galactose. The glucose is metabolized to lactic acid while galactose remains in the environment of the yoghurt (Goodenough and Klein 1976). Therefore, galactose

fermenting yeast strains in particular are known to cause spoilage of yoghurt (Caggia et al. 2001; Giudici et al. 1996).

Yoghurt is often added nuts, honey, preserved or dried fruit, containing sucrose and is a source of yeast infections. Yeast spoilage of yoghurt is seen as excessive gas production followed by swelling of the package, unpleasant yeasty odor and taste, changes in texture and color, and formation of visible yeast colonies (Caggia et al. 2001; Fleet and Yeasts 1998).

With intervals, studies of yeast in retail yoghurt have been reported from different countries. It is not unusual to detect yeast in yoghurt in numbers of 10^3 cells/g, and yeast count up to 10^8 cells/g has been reported. The contamination rate of yeast in yogurt were in the order of 10^3 for 20% of the examined samples in United Kingdom (Davis 1975) and Canada (Arnott et al. 1974).

The most common yeast species reported in yoghurt are *C. famata*/D. *hansenii*, *Pichia anaomala*, *C. versatilis*, *S. cerevisiae*, and *K. lactis*. These yeast are able to assimilate and ferment several of the main carbohydrates in plain and fruit yoghurt. They are also able to assimilate lactate and citrate. They grow at low pH-values and temperatures below 10°C. Further several are able to produce esterases and lipases, proteinases and peptidases hydrolyzing the milk fat and protein. Therefore the occurrence of yeast even in low numbers in yogurt will limit the shelf-life (Fleet 1990).

The occurrence and growth of yeast in yoghurt are closely related to poor hygiene and sanitation (Fleet and Mian 1987). As emphasized by Fleet (1990); Jakobsen and Narvhus (1996) spoilage should be prevented through implementation of general principles of good manufacturing practice. To extend the shelf-life of yoghurt the use of preservatives are permitted in some countries. However, yeast can be very tolerant to some preservatives. As an example *C. famata* and *K. marxianus* are still able to multiply in the presence of sorbate and benzoate at concentrations of 500 mg/l (Fleet and Mian 1987).

4.4 Kefyr

As a sign of immortality Allah granted a chosen tribe the first kefir grain and therefore the grains were called grains of the prophet. In another version of this tale the grains were given by the prophet Mohammed as a present as a sign of immortality together with the recipe of kefir, but it was forbidden to give away the grains or the recipe otherwise the grains would lose their healing power. These tales has been used as an explanation why the preparation of kefir was kept as a secret for so long. Even though, the history of this product is centuries old (Wouters et al. 2002) the first production of retail kefir was made in the former USSR in the 1930s (Wyder 1998).

Kefyr originates from Caucasus in Central Asia, but has become popular in many countries especially in the Middle East. Kefyr is an acidic and mildly alcoholic fermented milk beverage with a high level of CO₂. Traditional kefir is prepared in bags from goat hides, from either cow, sheep, or

goat milk inoculated with kefir grains (Motaghi et al. 1997). Kefyr grains are white, yellowish hard granules with shape as cauliflower. They are a result of a symbiotic microflora that are encapsulated in grains made of a fibrous matrix of coagulated casein, polysaccharides, fat and lysed cells. The polysaccharides in the kefir grains differ from other known polysaccharides and are called kefiran (Wyder 1998). The relative low temperature in the region of Caucasus where kefir originated led to a natural selection of mesophilic yeast, lactic and acetic acid bacteria in the kefir grains (Motaghi et al. 1997; Wyder 1998). Interactions between the micro-organisms have been reported (Koroleva 1988). The yeast stimulated the growth of the lactic acid bacteria releasing vitamins and amino acids and causing an increase in pH metabolizing lactic acid (Koroleva 1988). Several different composition of the microbiota in kefir grains has been detected. The composition depends on different cultivating techniques of the kefir grain, which is connected to the origin and temperature of the production site (Berger et al. 1999). The origin and temperature of the production site also have influence on the development of alcohol and CO₂ and then the final aroma and the viscosity of the kefir. The main yeast species detected in Kefyr are *K. marxianus*, *C. kefir*, *S. cerevisiae*, *S. delbrueckii* (Wouters et al. 2002). In the industrial production of kefir consistency in quality is a must. Therefore, retail kefir is seldom produced using kefir grains; instead commercial kefir cultures are based on a mixture of pure cultures isolated from kefir grains inoculated in milk. Commercial kefir cultures contain only yeast in very low concentration if any. Gas production by the yeast causes the package to blow and consumers seem to find swelling of the package undesirable in retail kefir.

4.5 Other Fermented Milk Products

Another well-known fermented milk produced with a natural yeast microflora is koumiss. Koumiss is like a milk wine with an alcohol percentage around 2 and a final pH about 4. In the beginning of the 1900 century koumiss was described as the greatest of the fermented milks as reviewed by Steinkraus (1996). Koumiss originates from Khazakstan and is traditionally made from mare's milk but variants made from cows milk has been reported (Steinkraus 1996). The dominating microflora in koumiss is thermophilic lactic acid bacteria and *Saccharomyces* spp. especially *S. unisporus* and *K. marxianus* (Montanari et al. 1996).

Yeast are also present in several indigenous African fermented milk products such as amasi/hodzeko from Zimbabwe (Gadaga et al. 2001), banik from Senegal (Gningue et al. 1991), rob from Sudan (Abdelgadir et al. 2001), nono from Nigeria (Okagbue and Bankole 1992), and ergo and ititu from Ethiopia (Gonfa et al. 2001). All traditional fermented milks are prepared by allowing raw milk to spontaneously ferment at ambient temperature. Lactic acid bacteria and yeast with *S. cerevisiae*, *K. marxianus*, *K. lactis*, and *C. kefir* as dominating yeast are part of the natural

microflora and it is assumed that yeast play an important role in the microbial interactions between the different micro-organisms and development of the characteristic aroma in these products. Traditional fermented milks are made in many households daily. These milks products represent an important source for human of energy, protein, vitamins, and minerals (Loretan et al. 1998). Because of the socio-economic changes that are taking place in Africa some of the traditional fermentation technologies might be lost together with the related microflora (Loretan et al. 1998). Therefore, interest in traditional African fermented milk is increasing, especially examination and identification of the high number of lactic acid bacteria and yeast and exploration of their role in the fermentation process of the milk (Loretan et al. 1998).

4.6 Cheeses

Yeast are detected and isolated from all sorts of cheeses (Bockelmann and Hoppe-Seyler 2001; de Boer and Kuik 1987; Nooitgedagt and Hartog 1988; Roostita and Fleet 1996a; Schmidt and Lenoir 1980; van den Tempel and Jakobsen 1998; Tzanetakis et al. 1998; Vivier et al. 1994; Welthagen and Viljoen 1998). The number of yeast can be in the range 10^4 cfu/g or even higher. The most common yeast species in cheese are *D. hansenii*, *Y. lipolytica*, *G. geotrichum*, *K. lactis*, *K. maxianus*, and *S. cerevisiae* with *D. hansenii* as the most predominant. The development of yeast in cheese occurs spontaneously while controlled use of yeast as starter cultures in cheese is used for production of some kind of mold ripened cheeses and smear ripened cheeses, but seldom for other types of cheese.

Until recently it was assumed that the yeast in cheese primarily originated from the cheese brine. Cheeses are often salted in brines containing 22–25% NaCl (Hansen et al. 2001) and often the brine is not changed or pasteurized between the salting of different batches leading to accumulation of salt tolerant yeast (Tudor and Board 1993) in the range of 10^2 – 10^6 cfu/g (Devoyod and Sponem 1970; Rohm et al. 1992; Seiler and Busse 1990; van den Tempel and Jakobsen 1998). It has also been suggested that the yeast in cheese originated from raw milk because of the survival of yeast through pasteurization (van den Tempel 2000; Vardillo et al. 1987). In a study carried out by Petersen et al. (2002) the successions of yeast on the surface of Danbo was followed using mtDNA RFLP. The investigation showed that the dominating flora after 4 days belongs to *D. hansenii* and that the dominating strain did not originate from raw milk, brine, or the starter culture, but from the dairy “house microflora,” which also includes yeast from air in the ripening room, ripening pads and humans. This indicates that cheese quality depends on an intact “house microflora” where the balance easily can be disturbed because the cheese surface in many ways is exposed to the environment of the dairy with its inherent population of micro-organisms (Bockelmann and Hoppe-Seyler 2001).

Examinations of different kinds of cheese showed that the yeast population often develops from a heterogeneous to a homogeneous population during the ripening period (Bockelmann and Hoppe-seyler 2001; Hansen et al. 2001; Petersen et al. 2002; van den Tempel and Jakobsen 1998). Yeast need an energy source to grow, therefore the ability to assimilate or ferment residual carbohydrates and acids is important for the yeasts in order to survive in the cheese and to compete with other micro-organisms during ripening (Roostita and Fleet 1996b). But also the general tolerance towards NaCl of yeasts and the effect of NaCl on the uptake of lactate and other carbohydrates is important (Petersen et al. 2002). The assimilation of different carbohydrates seems to be affected by the microenvironment in the cheese. Investigations of *D. hansenii* showed that the assimilation of lactate (Petersen et al. 2002; van den Tempel and Jakobsen 2000) and citrate (van den Tempel and Jakobsen 2000) was strain specific and that inhibitions for some strains occurred at 6% (w/v) NaCl while other strains could assimilate both citrate and lactate in the presence of 14% (w/v) NaCl (van den Tempel and Jakobsen 2000). Similar results were seen for assimilation of lactose and galactose. The same pattern was observed for *Y. lipolytica* though inhibition already occurred at low levels of salt, i.e., 2 % (w/v) NaCl for some of the strains examined (van den Tempel and Jakobsen 2000).

4.6.1 Yeast in Surface Ripened Cheese

Semi-soft and soft cheeses that develop a smear of microbial growth on the surface during maturation are called surface ripened cheese. These cheeses have maturation times that vary from several days to month and at temperature ranging from 10 to 20°C. Some examples of these cheeses are Limburger, Danbo, Brick, and Tilsiter (Fleet 1990; Bockelmann and Hoppe-Seyler 2001; Petersen et al. 2002). The microbial smear on the surface of these cheeses is very important for the maturation process and play a major role for the final cheese quality (Bockelmann 2002; Bockelmann and Hoppe-Seyler 2001; Fleet 1990; Jakobsen and Narvhus 1996). The cheese smear is a bio-mass of yeast and bacteria (Bockelmann and Hoppe-Seyler 2001; Fleet 1990; Fleet and Mian 1987; Masoud and Jakobsen 2003). The yeast species *D. hansenii* seems to be dominant and also the most important yeast during the whole maturation time but depending on the type of cheese other yeast such as *Trichosporon* spp., *Y. lipolytica*, *K. lactis*, and *Candida* spp. have been detected during the first day of ripening (Bockelmann 2002; Fleet 1990; Petersen et al. 2002). The bacterial flora consists of *Brevibacterium lines*, *Arthobacter* spp., *Corynebacterium* spp., *Micrococcus* spp., and *Staphylococcus* spp. (Bockelmann 2002; Bockelmann and Hoppe-Seyler 2001; Fleet 1990).

The microbial ecology of the smear is very complex, but during the first days of cheese ripening a natural selection takes place (Petersen et al. 2002). The most suitable group of yeast in the surface ripened cheese, seems to adapt easily to the microenvironment with high NaCl concentration, low pH,

and lactate as a main carbon source. Among the yeast *D. hansenii* in most cases grow fast and become the dominant species. It has been observed a particular subspecies out range the other strains and become dominant of *D. hansenii* present (Petersen et al. 2002).

The growth of *D. hansenii* on the surface of the cheese enhances the growth of the smear bacteria (Valdés-Staber et al. 1997) by metabolizing the lactate (Le Clercq-Perlat et al. 1999). After 4–7 days the pH increases from 5.2 to 5.7 allowing more acid-sensitive bacteria like *B. linens* to grow (Bockelmann and Hoppe-Seyler 2001; Petersen et al. 2002; Valdés-Staber et al. 1997). The number of yeast begin to decrease after one or two weeks of ripening while the bacteria dominates on the cheese surface during the last part of the ripening period (Bockelmann 2002; Petersen et al. 2002). Furthermore, yeast appears to support the bacterial growth by release of vitamins and amino acids (Viljoen 2001). Investigations carried out by Masoud and Jakobsen (2003) showed that *D. hansenii* had a significant effect on the intensity of the reddish pigment produced by the bacteria flora. Significant differences were observed between the *D. hansenii* strains examined but also the NaCl content and pH played a important role in the pigment production.

4.6.2 Yeast in Blue Mold Cheeses

Blue mold cheeses are semi-soft cheeses primarily ripened by growth of the mold *P. roqueforti*. Blue cheeses normally have a significantly higher content of NaCl compared to surface ripened cheeses and white mold cheeses. The NaCl concentration in blue mold cheeses after brining is 0.2% (w/w) in the core and 7% (w/w) in the surface layer. After eight weeks of maturation NaCl concentration is approximately 2.0% (w/w) in the core and 4.0% (w/w) in the surface layer. The pH after 24 hours is at the level pH 4.6–4.7. During ripening, pH in the core increases to about 6.5 and to 5.9 in the surface layer (Gobbetti et al. 1997; Godinho and Fox 1982; Hansen et al. 2001).

To permit air into enter the cheese center and carbon dioxide to escape the cheeses are pierced before maturation which also affect yeast growth. In blue veined cheese, yeasts are detected in high levels, without affecting cheese quality negatively (Fleet 1992; Hansen et al. 2001; van den Tempel 2000). The number of yeasts detected in blue mold cheese is in the order of 10^7 – 10^8 cfu/g on the surface and 10^5 – 10^6 cfu/g in the core, but higher concentrations have been observed (Hansen et al. 2001; van den Tempel and Jakobsen 1998). The most common yeasts isolated from raw milk cheeses like Roquefort are *D. hansenii*/C. *famata*, *C. catenulata*, *Y. lipolytica*/C. *lipolytica*, *C. krusei*, *T. cutaneum*, and *K. lactis*/C. *spherica* (Besançon et al. 1992; Roostita and Fleet 1996a). In Mycella, which is made from pasteurized milk, the predominant yeasts isolated from both the core and the surface is *D. hansenii*/C. *famata* but in the beginning of the maturation other yeast like *Zygosaccharomyces* spp., *G. geotrichum* *Y. lipolytica*, and *C. rugosa* are seen, in low

numbers (Hansen et al. 2001). In four-week old Danablu, the most common yeast are *D. hansenii* (van den Tempel and Jakobsen 1998). Examinations of Danablu produced at four different dairies and the Danish blue cheese Mycella showed that the yeast population developed from a heterogeneous flora to a homogeneous flora of *D. hansenii* during the ripening period (Hansen et al. 2001; van den Tempel and Jakobsen 1998).

Yeast are considered to play an important role in the ripening of blue mold cheese and seems to contribute positively to microbial environment by assimilation of the residual carbohydrates and acids. Yeast are assumed to create a stable microenvironment, which prevent undesired microbial growth. The gas produced in the curd during fermentation is likely to create minor fissures and chinks in the cheese curd, which is assumed to promote the development of *P. roqueforti* (Coghill 1979). Positive interactions have been detected between *D. hansenii* and *P. roqueforti* under conditions simulating the environment in Danablu (van den Tempel and Nielsen 2000). The yeast *S. cerevisiae* is known to have a positive affect on growth and sporulation of *P. roqueforti* (Hansen and Jakobsen 2001; Hansen et al. 2001). *S. cerevisiae* is also found to stimulate the release of free fatty acids (FFA) by *P. roqueforti* and a synergistic effect between *P. roqueforti* and *S. cerevisiae* has been demonstrated in the degradation of casein (Hansen and Jakobsen 2001; Hansen et al. 2001). The positive interactions between *P. roqueforti* and *S. cerevisiae* were verified in a dairy trial. In the cheeses added *S. cerevisiae* improved growth and earlier sporulation of *P. roqueforti* was observed compared to the reference cheeses. Furthermore, positive contribution from *S. cerevisiae* were also found in the aroma analysis, the degradation of casein, and by sensory analysis. The observed differences indicate the potential use of *S. cerevisiae* as an additional starter culture for production of Mycella (Hansen et al. 2001).

In laboratory studies inhibition of *P. roqueforti* by *Y. lipolytica*, *G. geotrichum* and *K. lactis* have been observed. A negative effect of the yeast on the growth of *P. roqueforti* has not been verified in cheese, but should be kept in mind if the development of *P. roqueforti* is slow or absent. It should also be keep in mind that some of these yeast, e.g., *D. hansenii* and *Y. lipolytica* are known to produce reddish pigment on the cheese surface primarily from oxidation of tyrosine to melanin (Carreira et al. 1998; van den Tempel and Jakobsen 2000).

4.6.3 Yeast in White Mold Cheese

White mold cheeses are semi-soft cheeses with growth of *P. camemberti* creating a white greyish mycelium on the surface on the cheese. The most famous variants are Brie and Camembert. In these cheeses pH is reduced to about 4.7 during the first 24 hours by the primary lactic acid starter culture. *P. camemberti* metabolizes the lactate to water and CO₂ resulting in an increasing pH, most pronounced on the surface of the cheese. A pH gradient of decreasing values will

be established towards the center of the cheese causing lactate to migrate towards the surface where it is used as a carbon source for *P. camemberti*. When all the lactate is depleted, casein will be degraded into amino acids and ammonia causing pH to increase further and the gradient to become stronger while the pH is still low in the center. The acid condition in the center causes soluble calcium phosphate to migrate towards the surface where it precipitates as a result of the higher pH (Karahadian and Linsa 1987; Vassel et al. 1986). The establishment of the pH gradient caused by *P. camemberti* indirectly is the key factor in the maturation process (Lawrence et al. 1987), but the desired soft texture of white mold cheeses is a direct result of the depletion of calcium phosphate in the center and the proteolytic activity of rennet, plasmin, and enzymes from the lactic acid bacteria and yeast. Yeast has been detected in several types of white mold cheeses and the positive role of yeast in the maturation and aroma formation of white mold cheese has been proposed (Schmidt and Lenoir 1980; Siewert 1986). However, the use of yeast as starter culture is still the exception rather than the rule in white mold cheeses. During ripening the yeast population increases to a level of 10^5 – 10^7 at the center and 10^6 – 10^8 at the surface. Several different yeast species have been isolated from white mold cheese and at the surface *K. lactis*, *K. marxianus*, and *G. geotrichum* have been the dominant cultures while *D. hansenii* has been predominant in the center. *S. cerevisiae* and *Zygosaccharomyces rouxii* are also found, but less frequently (Baroiller and Schmidt 1990). Except from *G. geotrichum* the role of yeast in the maturation process is still not clear, but it is assumed that their lipolytic and proteolytic activity and the capability to metabolize lactate and galactose, glucose and lactose play a role in the maturation. Further yeast have been mentioned to have an inhibitory effect on the undesired growth of *Mucor* spp. on the surface of Camembert cheese (Siewert 1986).

a. The Role of Galactomyces Geotrichum in White Mold Cheese. *Galactomyces geotrichum* is closely related to the production of white mold cheeses. *G. geotrichum* is often used as a co-culture together with *P. camemberti* in the production of white mold cheese (Addis et al. 2001; Molimard et al. 1995) and in a few variants of these cheeses *G. geotrichum* is used as the only culture. *G. geotrichum* is able to assimilate lactate and it grows faster on the surface of the cheese than *P. camemberti*. It seems to contribute strongly, along with different sulfides, to the characteristic aroma profile. Methanethiol and dimethyl is produced from methionine by two distinct pathways (Demerigny et al. 2000). The formation of dimethyl disulfide, dimethyl trisulfide, and *S*-methyl thioesters are also well known for this yeast (Berger et al. 1999). Further, *G. geotrichum* is able to produce volatile compounds like methylketones, alcohols, esters, and fatty acids (Jollivet et al. 1994).

Some strains of *G. geotrichum* have anti-microbial activities. Production and excretion of 2-hydroxy-3-phenylpropanoic acid, which have a broad anti-bacterial effect

(Dieuleveux et al. 1998) and D-3-phenyllactic acids inhibiting *Listeria monocytogenes* (Dieuleveux et al. 1998) have been reported. *G. geotrichum* is known to inhibit contaminating molds on the surface of mold cheese and in studies where *G. geotrichum* was cultured together with *P. commune*, *P. caseifulvum*, *P. verrucosum*, *P. solitum*, and *Aspergillus versicolor*; it was found that mycotoxin were produced in significantly lower concentration by the five molds compared to growth of the mold as single cultures (Nielsen et al. 1998a,b). All together, it indicates that *G. geotrichum* plays an important role in the inhibition of undesired microorganisms in mold ripened cheese (Nielsen et al. 1998a,b). Many strains of *G. geotrichum* have been described and the diversity among the strains is very pronounced with regard to all their technological characteristics (Spinnler et al. 2001).

4.6.4 Contribution and Spoilage of Yeast in Other Types of Cheese

Besides the important role yeast play in surface-ripened and mold-ripened cheese, they may also have a desired influence on the maturation and final quality of hard and semi-hard cheeses like Cheddar, Gouda, and Parmesan (Deiana et al. 1984; Viljoen and Greyling 1995). Yeast are mentioned to accelerate the ripening process (Deiana et al. 1984), but the role of yeast in these types of cheeses is not clear. A variety of yeast including, e.g., *D. hansenii*, *Y. lipolytica*, *S. cerevisiae*, *K. marxianus*, *C. catenulata*, *T. delbrueckii*, and *R. glutinis* have been isolated from these types of cheese and seems primarily to be connected to spoilage, e.g., uncontrolled maturation, flavor defects and blowing of the cheese, and undesired pigment formation. On the other hand, *D. hansenii* has been reported to have an inhibitory effect on the growth of *Clostridium tyrobutyricum* and *C. butyricum*, which is a well known spoilage organism in these types of cheeses (Deiana et al. 1984; Faticenti et al. 1983).

In unripened cheese yeast only seems to cause spoilage. In cottage cheese, spoilage caused by, e.g., *Y. lipolytica*, *C. lipolytica*, *Candidum sake*, *C. spherica*, and *K. marxianus* has been reported (Brockelhurst and Lund 1985; Fleet 1990). The spoilage appears as visible colonies on the surface of the cheese, flavor and aroma defects and undesired production of gas (Brockelhurst and Lund 1985).

Several investigations have been reported on yeast in Feta cheese (Kaminarides and Laskos 1992; Tzanetakis et al. 1998; Vivier et al. 1994). The dominant yeast in Greek Feta and the brine of Greek Feta is *D. hansenii*/*C. famata*, *S. cerevisiae*, *Torulaspora delbrueckii*, *Pichia farinosa*, and *Pichia membranaefaciens* (Kaminarides and Laskos 1992; Tzanetakis et al. 1998). In Danish and Sardinian Feta, uncontrolled gas production by yeast caused swelling defects in the final product (Fadda et al. 2001; Westall and Filtenborg 1998). In Danish Feta with swelling defects *Torulaspora delbrueckii* was predominant while the responsible yeast in Sardinian Feta was found to be *Dekkera anomala*. Several other yeast species such as *Y. lipolytica*, *D. hansenii*, *C. sake*, *K. marxianus*, *C. butyryi*, and *G. candidum*, *Dekkera*

bruxellensis, *K. lactis* were isolated from Feta cheese in these investigations. The yeast population in the Sardinian Feta was the same in the cheese from the two dairies investigated while the occurrence of yeast in Danish Feta seems to vary from dairy to dairy. The high concentration of yeast in the environment of the production area in the dairies indicated that the occurrence of yeast in these types of Feta was due to recontamination.

5 YEAST AS PROBIOTICS

According to the classical definition probiotic organisms regulate the microbial colonization in the digestive tract with a beneficial effect on human health (Gedek 1991). In relation to current and future probiotic products this traditional definition seems to be too narrow (Jakobsen and Narvhus 1996). The definition of a probiotic starter culture should be added a third-dimension, i.e., the extra nutritional-physiological values leading to a more general improvement of human health (Kurmman 1993; Lambelet et al. 1992). As such, the role of yeast as probioticum in dairy products has been overlooked. Studies of probiotic yeast as starter culture in dairy product, so far, has been very limited even though yeast occur in many dairy-related products. Historically, yeast as a probioticum has been linked with livestock feed and to the genus *Saccharomyces*. As reviewed by Gedek (1991) *S. boulardii* is a nonpathogenic yeast and is used both as preventive and therapeutic agent for the treatment of different diarrhoeal diseases (McFarland and Bernasconi 1993; Surawicz et al. 1989). *S. boulardii* was first isolated in Indonesia in the 1950s. In the fourth edition of the taxonomical study "The Yeasts" edited by Kurtzman and Fell (1998). *S. boulardii* is taxonomically classified under the name *S. cerevisiae*.

Among few studies on the use of probiotic yeast in dairy products to be mentioned is that Lourens-Hattingh and Viljoen (2001) investigated the potential of adding *S. boulardii* to yoghurt. The result showed that *S. boulardii* was able to survive four weeks at 4°C and even multiply to a level of about 10^6 in plain and fruit yoghurt. Gas and alcohol production were not observed, but it might be a constraint for incorporating *S. boulardii* into retail bio-yoghurt (Lourens-Hattingh and Viljoen 2001).

Several specific interactions between *S. cerevisiae* and enteric pathogens, e.g., *Escherichia coli*, *Salmonella*, and *Shigella* have been reported. Further, *S. cerevisiae* is reported to bind enterotoxin from enterobacteria to the surface of the yeast through a mannose-specific reaction (Gedek 1991). It has been reported that *S. cerevisiae* is able to survive the passage through the intestinal tract, with live cells detectable in the small intestine, which emphasize its potential as a probiotic.

Exploitation of killer factors as anti-microbial compounds could lead to another possibility for using yeast as probiotic. Killer toxins are proteins or glycoproteins secreted by the yeast cell and toxic to specific range of micro-organisms, e.g.,

enterobacteria (Brugier and Patte 1975; Polonelli and Morace 1986). Many yeast also produces metabolites, e.g., short-chain fatty acids known to be toxic against undesired micro-organisms in the intestinal tract (Gedek 1991).

Even though most yeast strains are considered to be safe, it is noteworthy that some strains found in dairy products, e.g., *C. catenulata*, *G. geotrichum*, and *C. kefir* are seen as opportunistic pathogens (Radosavljevic et al. 1999), and could represent a risk for human health especially for immunocompromised persons (Minervini et al. 2001; Radosavljevic et al. 1999).

6 CONCLUSION

The present study has documented the positive as well as the negative aspects of yeast in the dairy industry, and the focus on yeast in dairy products seems to be more intensive than ever.

The negative role of yeast as spoilage organisms is still a problem and seen in all kind of dairy products. The most effective way to solve this problem is improvement of hygiene and sanitation, and tighter specifications for various ingredients, e.g., for yoghurt.

The technological characteristics of important yeast and the complexity of the microbiota of many dairy products have been discussed in several recent publications. Especially, the understanding of the positive role yeast play in maturation of surface-ripened cheese is increasing, but only to a very limited extent brought to the point of active use of yeast, i.e., the application of yeast as starter culture. New information on the technological characteristics of yeast and the diversity among strains of the same species is providing a better background for the selection and use of yeast as starter cultures in dairy products. The advantages of deliberate yeast addition to fermented and matured dairy products are evident. The advantages are mainly related to the proteolytic and lipolytic activity of yeast, their influence on aroma formation, and their acceleration of the ripening process. Further, yeast play an important role in microbial interactions resulting in improved performance of starter cultures and inhibition of undesired micro-organisms. Yeast also have probiotic properties.

The taxonomy for yeast is very complex. New methods, including molecular techniques are used increasingly, but for many of the yeast genera related to dairy products the molecular methods are not yet fully developed or sensitive enough to distinguish yeast strains at species or subspecies level. However, these techniques are a valuable supplement to the traditional methods of identification and powerful tools in ecological studies including the microbiota from indigenous fermented dairy products and typing of starter cultures. New approaches for developing methods for typing yeast with potential as starter culture and characterization of yeast causing spoilage in dairy product should be given high priority in future research together with development of yeast starter cultures and the use of yeast as probiotics.

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Flavors and Aromas

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1 INTRODUCTION

The demand for natural flavoring substances has increased sharply in recent years, and tremendous interest has been shown in their production by microorganisms (Berge and Evenhuis 1998; Carina 1995; Schindler and Schmid 1982; Schreiber et al. 1997). Fungal biotransformation of low-cost substrates to flavor and aroma compounds having a high value appears to have great commercial potential. Such compounds have been accepted by the United States Drug Administration (USDA) and by the European Flavour Commission (88/388/EWG 1988; 91/71 EWa and 91/2 EWG 1991) as being natural and falling under the generally regarded as safe (GRAS) category (Janssens et al. 1992; Taylor and Mottram 1996). The flavor products obtained by microbial routes are optically pure, extracellular, and suitable for commercial exploitation because of easy down-stream processing and high yields. Extensive reviews on this subject have been published by Demyttenaere (2001); Hagedorn and Kaphammer (1994); Mestri (1994); and Van der Werf et al. (1997). This chapter will deal with the description and discussion on fungal flavors and aromas.

2 PRODUCTION OF MICROBIAL FLAVORS

Flavoring chemicals are produced either by biotransformation of precursor compounds to flavour end-products, or by *de novo* synthesis. Fungi play an important role in this field, as they can transform abundantly available substrates such as terpenes, and thereby make the process economical.

2.1 Terpenoids

Terpenoids, constitute one of the largest groups of natural products and impart a wide variety of pleasant and floral scents. These compounds contain one, or more, basic isoprene units which are joined head to tail. Depending on the number of units, terpenes are classified as monoterpenes (2 units), sesquiterpenes (3 units), diterpenes (4 units), triterpenes (6 units), and polyterpenes (higher units). Terpenoids, produced from filamentous fungi are used by various food industries. Readily available monoterpenes, such as α -pinene and limonene, are used as substrates for conversion into flavoring compounds (Berger et al. 1992; Van der Werf et al. 1997).

2.1.1 Monoterpenes

a. Limonene Conversion. Limonene can be converted to major products like carveol and carvone by *Penicillium italicum* and *P. digitatum* (Bowen 1975). When limonene concentration was increased there was a decrease in the quantity of end-products produced. Addition of sucrose increased microbial growth but the conversion was low. The conversion of limonene to carveol seems to be a single-step reaction, involving addition of a hydroxyl group at C-3 (Figure 1). Limonene biotransformation was first carried out by Rama Devi and Bhattacharyya (1978) who studied the oxygenative and prototropic molecular rearrangements during terpene transformation by *A. niger*.

Abraham et al. (1985) investigated the biotransformation of (R)-(+)-limonene to α -terpineol by *P. digitatum*. DSM 62840. Abraham et al. (1986) studied microbial transformation of terpenoids with 1-*p*-menthene; they used *Corynespora cassiicola* and *Diplodia gossypina* to convert (S)-(-) and (R)-(+)-limonene, α -terpinene, γ -terpinene, and

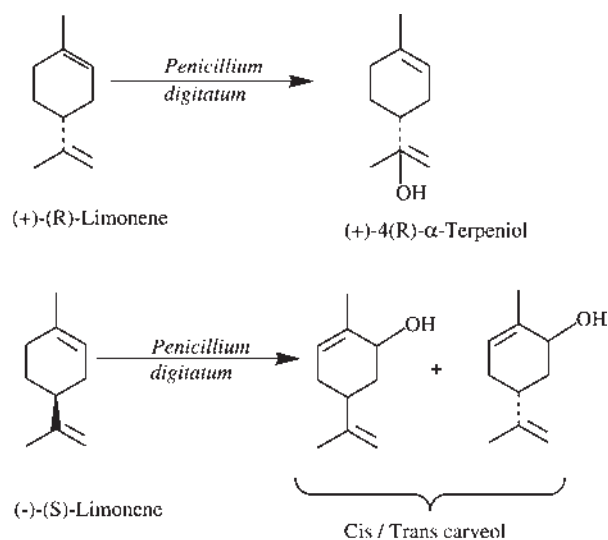


Figure 1 Biotransformation of limonene.

terpinolene to 1,2-*trans*-diols. They found that the intermediary epoxide could be cleaved by the hydroxyl groups present in the substrates. Noma et al. (1992) employed *Aspergillus cellulosa* to transform limonene to major products like carveol, perillyl alcohol, and α -terpineol. They reported the possibility of introducing an oxygen functional group into limonene at the C-3 position, utilizing citrus peel oil as C source. Demyttenaere et al. (2001) used a solid phase micro-extraction technique to study the conversion of limonene by *P. digitatum*, to obtain α -terpineol as the major product. For details refer to the review by Van der Werf et al. (1997), which deals with terpene biotransformation, and

with the problems related to commercialization of terpenoid products. Rensburg et al. (1997) have shown the possibility of limonene biotransformation in yeasts.

b. Pinenes. The biotransformation of α -pinene has considerable commercial importance. Hydroxylation of α -pinene by *A. niger* has been reported by several workers (Bhattacharyya et al. 1960); as also the references in their papers. Verbenol, verbenone, and sobrerol were obtained on biotransformation of α -pinene (Bhattacharyya et al. 1960). Verbenone was probably a product of auto-oxidation, whereas verbenol was formed by microbial oxidation (Figure 2; Bhattacharyya and Ganapathy 1965). Rama Devi and Bhattacharyya (1978) studied the conversion of α -pinene to 1-*p*-menthane, involving rupture of the cyclobutane ring in the bicyclic system.

Agrawal (1999) used an UV auxotroph of *A. niger* to increase the yield of verbenol from 10% obtained with a wild type to 25%. The enzyme α -pinene hydroxylase involved in α -pinene conversion to verbenol was NADPH dependent, and could be stabilized for 3 days using sorbitol along with DTT (Nazhat-ul-Ainn and Agrawal 2002). No biotransformed products from α -pinene were found under nitrate reducing conditions (Pavlostathis and Misra 1999). Optimization of growth conditions and media conditions can enhance the yield of verbenone (Agrawal and Joseph (2000a)) from α -pinene using *Penicillium* sp. Major biotransformations of some terpene substrates to flavoring compounds are given in Table 1.

2.1.2 Other Compounds

Many commercially important flavouring constituents, like esters, lactones, aldehydes, ketones, tobacco flavorings, and alcohols are produced with the help of fungi.

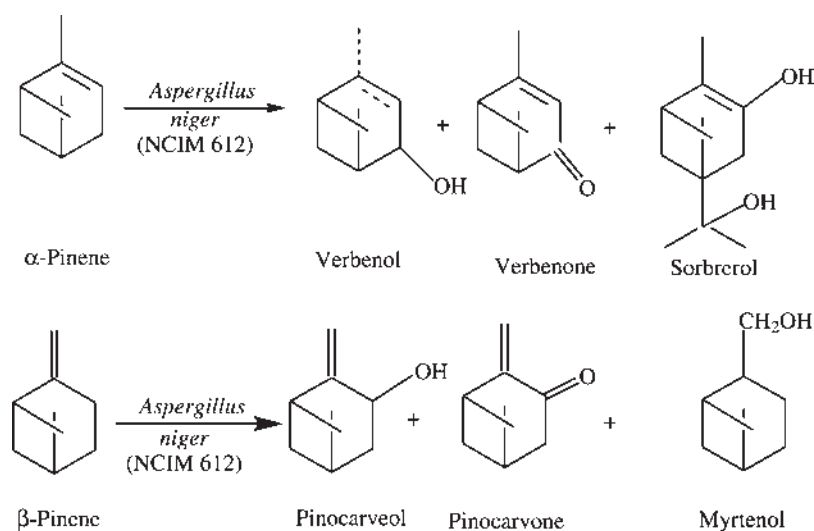


Figure 2 Biotransformation of pinenes.

Table 1 Fungal biotransformation of terpene substrates to high valued flavoring compounds

Fungi	Substrate	Products	References
<i>P. italicum</i>	Limonene	Carveol, carvone	Bowen (1975)
<i>A. cellulosa</i>	Limonene	Carveol, perillyl alcohol, and α -terpineol.	Noma et al. (1992)
<i>Y. lipolytica</i>	Limonene	Perillic acid, 7-hydroxy piperitone	Rensburg et al. (1997)
<i>P. digitatum</i>	Limonene	α -Terpineol	Demyttenaere et al. (2001)
<i>A. niger</i>	α -Pinene	Verbenol, verbenone	Prema and Bhattacharyya (1962)
<i>Penicillium</i> sp.	α -Pinene	Verbenol	Agrawal et al. (1999)

c. Ester. Various aliphatic esters could be obtained from lyophilized, whole cells of *Rhizopus oryzae*; such cells can tolerate high substrate concentrations, and hence allow the production of large amounts by semi-continuous or continuous addition of the substrate, e.g., geranyl butyrate (Molinari et al. 1995). Lyophilized whole cells of *R. Delemere* were utilized to catalyze direct esterification of primary alcohols (*n*-hexanol) to give a very high yield (98%) of hexyl caprylate (Molinari et al. 1998). Agrawal et al. (2000) have demonstrated the production of dihydrocarvyl acetate from nerol, using *Mucor* sp. Although the metabolic pathway was not studied, it appears that ring closure through geranyl pyrophosphate led to the formation of dihydrocarvone, which may then be reduced to form dihydrocarveol and then acetylated to form dihydrocarvyl acetate. Regio-specific esters were obtained by using *A. niger* to form acetates of citronellol, geraniol, and linalool (Madhyastha 1988). Patel et al. (1992) described a simple method of utilizing *Geotrichum candidum* to improve the optical purity of (*S*)-(-)-4-chloro-3-hydroxy butanoic acid methyl ester by converting it to 4-chloro-3-oxobutanoic acid methyl ester. Cell extracts contained a single enzyme that catalyzed the reduction to the hydroxy product. Farbood et al. (1987) demonstrated the synthesis of terpene esters through an amino acid precursor in *G. fragrans*. Gatfield (1988) worked out the possibility of ester synthesis from lipase enzyme in *Mucor michei*; this process could improve upon the isolation and purification steps, when compared to the aqueous fermentation systems.

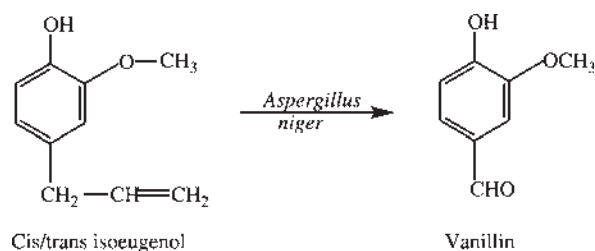
d. Lactones, Aldehydes, and Ketones. Biogenesis of volatile lactones from fungi has proved to be industrially successful (Cardillo et al. 1990). Lactone formation appears to be the result of a metabolic overflow. When the regular 3-hydroxylation of fatty acids is expanded to 4- or 5-hydroxylations, lactone formation takes place. These compounds impart fruit-like, buttery, sweet, or nutty odors.

Macrocyclic musks were produced from *Ustilago zaeae*, using ustilagic acids as precursors (Gatfield 1988). Using octanoic acid, Gregory and Eilerman (1989) accomplished the bioconversion in *Mucor* sp. to delta-gamma octalactone. Farbood et al. (1990) obtained a mixture of saturated and unsaturated γ -decalactones using γ -keto acid as the substrate; the acid is reduced to γ -hydroxy acid, which ultimately cyclizes to γ -lactones. Serrano-Carreón et al. (1992) studied

the formation of γ - δ - and δ -hydroxy acids in *Trichoderma harzianum*. *Sporobolomyces odoratus* culture was able to produce an intense peach (γ -decalactone) and mutton (*cis*-6-dodecen-4-olide) odors (Lee and Chou 1994). *P. roqueforti* was employed for producing lactones, mainly 4-dodecanolide from hydrolyzed oils like soybean and copra (Chalier and Crouzet 1992). The compound, 4-dodecanolide is formed by γ -hydroxylation of the corresponding saturated acid, or by β -oxidation of oleic acid into 3-dodecanoic acid, followed by lactonization of the mentioned acids. The biosynthetic pathway in *S. odoratus*, which results in the formation of 4-hexanolide as an oxidation product of linoleic acid, was studied by Taylor and Mottram 1996. It is possible that lactone is the result of β -oxidation of linoleic acid to 3,6-dodecadienoic acid, which is later hydrated and lactonized.

Production of 6-pentyl- α -pyrone by *Trichoderma viride* has been reported (Prapulla and Karanth 1992). The addition of amberlite XAD-2 resin to the medium overcomes product inhibition. Kalyani et al. (2000) studied the formation of 6-pentyl- α -pyrone in surface cultures of *T. harzianum*, under submerged conditions. Characterization of 6-pentyl- α -pyrone also has been done using *T. koningii* (Benoni et al. 1990).

Aldehydes are usually formed via a Strecker degradation of amino acids. This involves oxidative deamination and decarboxylation of α -amino acids, leading to the formation of an aldehyde containing one carbon atom less than the original amino acid (Mottram 1994). Fungi have shown great potential in producing high yields of these flavoring compounds. Among aldehydes, benzaldehyde (almond aroma) and vanillin, are widely used by food industries. *Cis/trans*

**Figure 3** Biotransformation of isoeugenol.

isoeugenol (1:4) was converted by *A. niger* into vanillin (Figure 3; Robenhorst and Hopp 1991).

Berger et al. (1987) studied the formation of a methoxy benzaldehyde in *Ischnoderma benzoicum*. Casey and Dobb (1992) reported the formation of aromatic aldehydes from aromatic amino acids, via a phenylpyruvic acid like benzaldehyde, using *Trichosporon beiglii*. Ketones are characterized by the presence of a carbonyl group, and are classified as aliphatic, aromatic, or phenol derivatives. They are synthesized by fungi in response to the presence of short-chain fatty acids, or as a means of recycling of COA. Mestri (1994) has discussed the production of nootkatone from valencene by an oxidation process involving *P. camemberti* and *A. niger* (Figure 4).

Biotransformation of nerol, geraniol, and citral, in surface cultures of *A. niger* and *P. digitatum*, to 6-methyl-hept-5-en-2-one (92–99%) has been studied by Demyttenaere and DePooter (1996) and Demyttenaere et al. (2000). These workers indicated an oxidative pathway wherein the alcohol is oxidized to aldehyde, and then to 6-methyl-hept-5-en-2-one, with no intermediary products. The pathway of this biotransformation of geraniol into 6-methylhept-5-en-2-one by *P. digitatum* has been elucidated recently by Wolken and Van' der Werf (2001), who also point out that citral is converted into geranic acid in this process. Furthermore, they also detected a novel enzymatic activity, wherein citral lyase converts citral to methylheptenone and acetaldehyde, independently of cofactors.

e. Ionone. Tobacco flavor is obtained by the transformation of ionone compounds. These compounds are widely distributed in nature, and are the constituents of many essential oils. Fungi, such as *A. niger*, converted ionone to α -cyclohomogeraniol, 3-oxo- α -cyclohomogeraniol, and benzofuran, as depicted in Figure 5 (Krasnobajew and Helmlinger 1982).

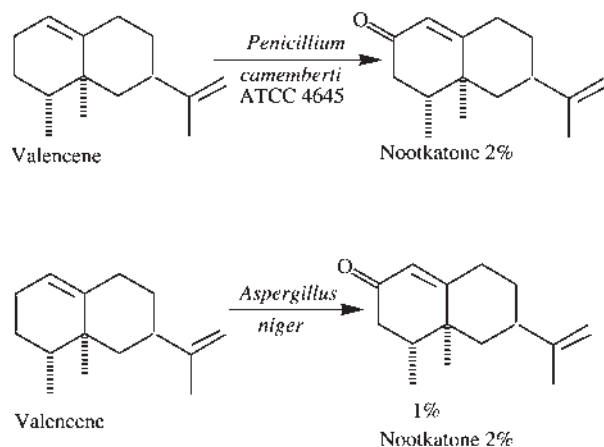


Figure 4 Biotransformation of valencene.

Larroche et al. (1995) have reported the formation of hydroxy and exo derivatives from β -ionone by *A. niger*. The recovery of the products was 100%, after 230 h of cultivation. This work paved the way for a possible fed-batch procedure, without replacement of the medium. When β -ionone was the only carbon source in the medium it stopped fungal growth, and was converted into hydroxy metabolites, probably by the action of a hydroxylase system. Grivel et al. (1999) made a dynamic model for biotransformation of β -ionone in *A. niger*. As the precursor is less soluble in water, it gave rise to a two-phase liquid system with high volatility and poor chemical stability. The products were 5,6-epoxy-5,6-dihydro- β -ionone, dihydro actinidiolide, and 4-oxo- β -ionone, with a molar yield of 32%; a high loss by stripping is a serious drawback of this process.

f. Acid Formation. The yields of acid produced by fungi are found to be commercially feasible, and are being utilized by industries. Armstrong et al. (1989) reported high yields of citric acid, which is perhaps the best known flavor compound produced by *A. niger*. Fabritius et al. (1998) studied the conversion of palmitic acid to (*R*)-(*Z*)-3-hydroxy-9-octadecendioic acid in *Candida tropicalis*; when ricinoleic acid was utilized as the sole carbon, optically pure (*R*)-(*Z*)-7-hydroxy-9-octadecenedioic acid was obtained. As only one regio-isomer was obtained, the hydroxylation was regiospecific. Fatty acids are, therefore of great interest to the chemical industry, as they provide a new avenue for commercial exploitation.

g. Alcohols. Various alcohols, which are utilized widely as flavors are produced by fungal conversions. The formation of sclareol, a labdane diterpene used in foods, has been studied in *C. albidus* (Farbood et al. 1986). Bioconversion of citronellol, leading to the formation of 2,6-dimethyl-1,8-octanediol and (*E*)-2,6-dimethyl-2-octene-1,8-diol was studied with four strains of *Botrytis cinerea* in grape must (Brunerie et al. 1987). The substrate was metabolized to the ω -hydroxylation product (*E*)-2,6 dimethyl-2-octene-1,8-diol and 2,6-dimethyl-1,8-octanediol. Regiospecific esters were produced by *A. niger* from acetates of citronellol, geraniol and, linalool (Madhyastha 1988). The major product formed from citronellol acetate, was citronellol. Geraniol was produced from geranyl acetate while linalool and 8-hydroxy linalool were the major compounds from linalyl acetate. A bioreactor, based on an aqueous/organic two-phase system, was designed by Doig et al. (1998) for the biotransformation of baker's yeast to geraniol. Uzura et al. (2001), using resting cells of *Fusarium moniliforme*, and propyl benzene as substrate, was able to form 1-phenylpropanol with high regio- and stereo-specificity. Lomascolo et al. (2001) worked out the possibility of producing 2-phenylethanol (rose flavor) from *A. niger*, using as precursor phenyl alanine, which was synthesized as the sole aromatic product. Some examples of nonterpene fungal flavoring compounds are given in Table 2.

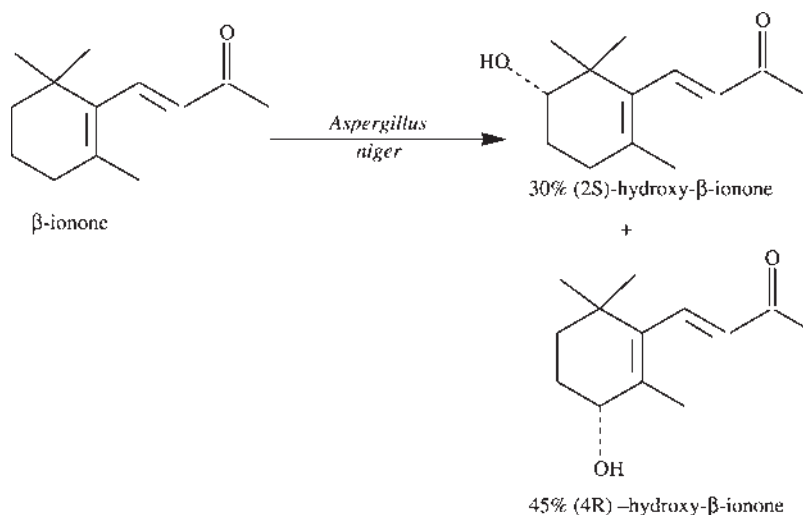


Figure 5 Biotransformation of ionone.

2.2 De Novo Synthesis

Unlike in the case of biotransformation, in *de novo* synthesis no specific precursors are needed in the medium. Products formed by using different micro-organisms include lactones, esters, and oxygenated terpenes (Table 3; Schindler and Schmid 1982; Scharpf et al. 1986).

The fruity flavor and aroma compounds produced by *de novo* synthesis depend on the media constituents like glucose, amino acids, and salts (Quehl and Ruttloff 1992). In the *de novo* synthesis category most of the experiments carried out till now, were only on a laboratory scale, and the product yields were found to be low. However, they have helped in the study of the enzyme systems involved, and of their reaction pathways during the synthesis. Sarris and Latrasse (1985) demonstrated when *Fusarium poae* was grown on a solid malt medium till sporulation, it produced a lactone with a peach-like aroma; but under insufficient aeration 2-methylbutanol and 3-methylbutanol were produced. *T. viride* on a simple growth medium generated a

strong coconut aroma of 6-pentyl-2 pyrone (Welsh et al. 1989).

Composition of the medium and age of culture generally influence the formation of flavoring compounds in *de novo* synthesis. For instance, an intense banana aroma was formed by *Ceratocystis fimbriata* when the growth medium was supplemented with several nutrients (Christen and Rainbault 1991). Similarly, Lee et al. (1999) also reported formation of γ -decalactone, in an optimized immobilized culture of *Sporidiobolus salmonicolor*.

2.3 Complex Flavors

Sharpell (1985) has discussed in detail some complex mixtures of flavors and fragrances, which are associated with natural products. Microbial processes appear to be very promising for the production of complex dairy and mushroom like flavors. Screening of different organisms made it possible

Table 2 Nonterpene fungal flavoring constituents

Compound	Fungi	Products	References
Ester	<i>R. delemere</i>	Aliphatic esters	Molinari et al. (1998)
Lactones	<i>S. odorus</i>	4-Decalactone	Lee and Chou (1994)
	<i>T. viride</i>	6-Pentyl- α -pyrone	Prapulla and Karanth (1992)
Aldehyde	<i>I. benzoicum</i>	Methoxy benzaldehyde	Berger et al. (1987)
	<i>T. beigelii</i>	Benzaldehyde	Casey and Dobb (1992)
Acid	<i>C. tropicalis</i>	3-Hydroxydioic acid	Fabritius et al. (1998)
Alcohol	<i>B. cinerea</i>	Octanediol	Brunerie et al. (1987)
	<i>A. niger</i>	2-Phenylethanol	Lomascolo et al. (2001)

Table 3 De novo synthesis of fungal flavoring constituents

Fungi	Odour attribute	Products	References
<i>T. viride</i>	Coconut	6-Pentyl-2-pyrone	Prapulla and Karanth (1992)
<i>G. candidum</i>	Quince-like	Ethyl ester	Latrasse et al. (1987)
<i>F. poae</i>	Coconut	γ -Lactone	Sarris and Latrasse (1985)
<i>A. niger</i>	Sour	Citric acid	Armstrong et al. (1989)
<i>A. oryzae</i>	Pine-like	1-Octen-1-ol	Scharpf et al. (1986)
<i>A. terreus</i>	Fruity	Ethyl acetate	Schindler and Schmid (1982)

to identify some of the organisms, which produced complex flavors (Gatfield 1988).

2.4 Dairy Flavors

Dairy flavors, primarily cheese flavors, are widely used in the food industry. Kristofferson (1973) has studied the biogenesis of cheese flavor. Maga (1974) investigated the formation of flavor constituents by using *Penicillium* sp. on milk and bread. Similarly, formation of cheese flavor, either by the enzymatic or the chemical route, has been studied by Law and Mulloholand (1991) who used fungal cultures.

2.5 Mushroom Flavors

Sugihara and Humfeld (1954) who found that mushroom flavor could be produced by *Lepiota rhacodes* when grown under submerged conditions. Gilbert (1960); Litchfield et al. (1963); and Le Duy et al. (1974) studied the production of mushroom flavor by *Morchella crassipes*. *Lentinus edodes* produced 1-octen-3-ol, 5'-amp, an intense mushroom flavor when the medium was supplemented with ethanol (Sugimori et al. 1971). Hamid et al. (1972) investigated the production of mushroom flavor from *Trichoderma nudum* under submerged conditions, while Van Eybergen and Scheffers (1972) reported its production in the mycelium of *Boletus edulis*. Dijkstra (1976), Pyssalo and Honkanen (1976), and Card and Avisse (1977) found that the fermentation conditions as well as media constituents play a vital role in the production yield. Mushroom flavors have also been obtained from *A. oryzae* (Scharpf et al. 1986), *Caprinus micaceus*, *Merulins rufus*, and *Poria vaillantii* (Schindler and Schmid 1982).

3 COMMERCIAL IMPORTANCE

Production of flavoring compounds using fungal cultures is economical and industrially viable. Presently, many flavoring compounds are being produced industrially by the use of fungal cultures; e.g., (R)-8. Dodecanolide using yeast by

Unilever Chemical Company UK; ethyl butyrate by Hercules, USA (Dziezak 1986); menthol by Nippon Mining Company, Japan (Watanabe and Inagaki 1978); and macrocyclic musk by Quest International (Jeffcoat and Willis 1988).

4 CONCLUSIONS

In view of the increasing demand for natural flavors, their production by microbial means seems to be a good preposition. The method is efficient, effective, and also economical. Monoterpene substrates like limonene and alpha-pinene, which are produced in large quantities in nature, can be used for the production of flavoring compounds of high value. The product yields obtained are high enough for industrial production, and many microbial processes are already operating. Newer genetic engineering techniques may still help in enhancing the product yield. There is also a need for metabolic pathways to be studied in detail so that high product yields can be obtained by its regulation. There is also need to investigate the effects of different parameters on a pilot plant scale which will lead to many more successful commercial technologies.

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Antifungal Food Additives

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1 INTRODUCTION

The problem of food spoilage has plagued humans throughout history. As early humans evolved from a gathering and hunting life style to raising crops and keeping animals, they were forced to store their own food. Early attempts to preserve foods involved use of sugars, spices, salts, and wood smoke. Today, however, preservation has used such factors as temperature, water activity, pH, gases, organic acids, salts, antibiotics, irradiation, packaging, and various combinations of these factors. No matter which factors are selected, use of the proper antimicrobial is dependant on the chemical properties of the antimicrobial; properties and composition of the food product; type of preservation system, other than the chemical, used in the food; type, characteristics, and number of microorganism; safety of the antimicrobial and cost effectiveness of the antimicrobial (Ray 2001).

Fungi are among the most challenging organisms to inhibit in foods given their ability to grow under a diverse range of environmental conditions. Environmental conditions such as water activity (a_w), pH, temperature, and atmosphere can be manipulated to control fungal growth. However, these conditions often need to be taken to extremes to control fungi, since subgroups exist that have become adapted to extreme environmental conditions. Using water activity as an example, most molds are inhibited by an a_w of 0.80 or lower, although some xerophilic molds can grow at a_w values as low as 0.65. Most yeast are inhibited by a_w values of 0.87, but some osmophilic yeasts can grow at a_w values as low as 0.60 (Farkas 1997; ICMSF 1980).

Most fungi are little affected by pH over broad range, commonly 3–8. Some molds can grow at pH 2.0, and yeasts at pH 1.5 (ICMSF 1980; Rahman and Labuza 1999). However, as pH moves away from an organism's optimum growth range, typically about 5.0 for fungi, the effect of other growth-limiting factors becomes more apparent.

Fungi are generally easily inactivated by heat treatments such as pasteurization, although some heat-resistant molds associated with fruits and fruit products can survive rather severe heat treatments and spoil products such as pasteurized juices and canned fruits. In addition, molds can grow over a wide range of temperature. Some mold can grow at temperatures less than 0°C while other can grow at 50–55°C.

Molds have an absolute requirement for oxygen. Many species, however, are efficient oxygen scavengers and can grow in atmospheres containing less than 1.0% O₂ (Cerny 1979). From a practical standpoint, it can be difficult to inhibit mold growth in foods solely by exclusion of oxygen from the package. Foods often contain dissolved oxygen, which slowly equilibrates with the package headspace, and oxygen can leach through all but the most impermeable packages. While mold growth can be delayed, it is not inhibited over long-term storage. Yeast have no requirement for oxygen and can grow in its complete absence.

Antifungal food additives are an efficient, cost-effective, and often the only successful way to control fungal growth in foods. Antifungal food additives are basically chemicals that prevent or interfere with mold growth. These chemicals may be found naturally occurring in certain foods, such as some organic acids and essential oils, or may be added to food during processing (Naidiu 2000; Thompkin and Singh 2000). The various antifungal food additives are briefly overviewed in this chapter.

2 ORGANIC ACIDS

Organic acids have been used for years to control fungal spoilage of foods. They find wide use because of solubility, taste, and low toxicity. The mode of action of organic acids is attributed to depression of intracellular pH by ionization of the undissociated acid molecule or disruption of substrate

transport, by alteration of cell membrane permeability. In addition to inhibiting substrate transport, organic acids may inhibit NADH oxidation, thus eliminating supplies of reducing agents to electron transport systems (Doors 1993; Liewen and Marth 1985).

Since, the undissociated portion of the acid molecule is primarily responsible for antifungal activity, effectiveness is dependant upon the dissociation constant of the acid and pH of the food to be preserved. Because the dissociation constant of most organic acids is between pH 3 and 5, organic acids are generally most effective at low pH values. This along with solubility properties determines the foods in which organic acids may be effectively used.

A few fungal species possess mechanisms of resistance to organic acid preservatives. *Saccharomyces baili* is resistant to high concentrations of sorbic and benzoic acids (Warth 1977). Some molds in the genus *Penicillium* can grow in the presence of high concentrations of sorbic acid and decarboxyated sorbic acid to 1,3-pentadiene, a volatile compound with an extremely strong kerosene-like odor (Liewen and Marth 1984; 1985; Tsai et al. 1988). When resistance to or metabolism of an organic acid is a problem, other preservative systems must be used.

2.1 Sorbic Acid

Sorbic acid and its potassium salt are the most widely used forms of this compound and are collectively known as sorbates. The salt forms are highly soluble in water, as is true for all organic acids. Their most common use is preservation of food, animal feed, cosmetic, and pharmaceutical products, as well as technical preparations that come in contact with the human body. Methods of application include direct addition into the product; dipping, spraying, or dusting the product; or incorporation into the wrapper (Ranun 1999).

Typical use levels in foods range for 0.02% in wine and dried fruits to 0.3% in some cheeses (Table 1). Food in which sorbate has commercially useful antimicrobial activity

Table 1 Typical concentration (%) of sorbic acid used in various food products

Cheeses	0.2–3.0
Beverages	0.03–0.10
Cakes and pies	0.05–0.10
Dried fruits	0.02–0.05
Margarine	0.05–0.10
Mayonaise	0.10
Fermented vegetables	0.05–0.20
Jams and jellies	0.05
Fish	0.03–0.15
Semimoist pet food	0.1–0.30
Wine	0.02–0.20
Fruit juice	0.05–0.20

Source: Liewen and Marth (1985).

include baked goods (cakes and cake mixes, pies and pie fillings, doughnuts, baking mixes, fudges, and icing), dairy products (natural and processed cheese, cottage cheese, and sour cream), fruit product (artificially sweetened confections, dried fruit, fruit drinks, jams, jellies, and wine) vegetable products (olives, pickles, and relishes salads), and other miscellaneous products (certain fish and meat products, mayonnaise, margarine, and salad dressings) (Sofos and Busta 1993).

Environmental factors such as pH, water activity, temperature, atmosphere, type of microbial flora, initial microbial load, and certain food components, singly or in combination can influence the activity of sorbate. Together with preservatives such as sorbic acid, they often act to broaden antimicrobial action or increase it synergistically. Use of other preservatives in combination with sorbate can broaden or intensify antimicrobial action. If growth of spoilage or pathogenic organisms is inhibited, but the microorganism is not killed, growth will eventually resume under proper conditions. The length of inhibition will vary with storage temperatures as well as with any of the other factors discussed.

Sorbic acid is a broad-spectrum antimycotic that is effective against yeast and molds. The antifungal effect of sorbate is greater at pH less than 5.0. Sorbic acid has little antifungal activity at pH values higher than 5.6. Above this pH, little of the acid is in the antimicrobially active dissociated form. However, sorbic acid has a relatively high dissociation constant compared to benzoic or propionic acids and is, therefore, usually the most effective of the organic acids at pH levels of 5.0 or higher. This is presented in Table 2 (Luck and Jager 1997; Ray and Bullerman 1982).

2.2 Benzoic Acid

Benzoic acid also has widespread use in the food industry. It occurs naturally in raspberries, cranberries, plums, prunes, cinnamon, and cloves (Doors 1993). As an antifungal food additives, the water-soluble sodium and potassium salts and the fat-soluble acid form are suitable for food and beverages with a pH below 4.5. Benzoates have little effect at neutral pH values. They are not as effective as sorbates at pH 5.0 (Table 2), but their effectiveness increases at lower pH values.

Table 2 Minimum concentration (%) of preservative required for inhibition of mold growth at pH 5.0

Compound	Species		
	<i>A. soini</i>	<i>P. citrinum</i>	<i>A. niger</i>
Benzoic acid	0.15	0.20	0.20
Propionic acid	0.06	0.08	0.08
Sorbic acid	0.02	0.08	0.08

Source: Liewen and Marth (1984).

Benzoic acid is active against yeasts and molds, including aflatoxin-forming microorganisms (Ray and Bullerman 1982). The acid form is often added to the fat phase and the sodium salt to the water phase of product such as salad dressings, mayonnaise, pickled vegetable, fruit products, and fruit drinks. Because benzoate can impart a fairly strong bitter off-flavor, it is frequently used in combination with sorbate. This mixture is often more effective in inhibiting yeast and molds than a comparable level of either preservative alone (Luck and Jager 1997). In addition, the mixture is less offensive organoleptically than benzoate alone.

2.3 Propionic Acid

This organic acid inhibits molds but not yeasts. It occurs in some foods as a result of natural processing. It is present in Swiss cheese at concentration up to 1%, where it is produced by the bacterium *Propionibacterium shermanii* (Beuchat 2000). Since, yeast are typically unaffected, the acid can be added to bread dough without interfering with leavening (Ranun 1999).

In the food industry, propionic acid is often used as a sodium or calcium salt (Ray 2001). Propionates are used primarily to inhibit molds in bakery goods. In addition to their antimycotic properties, propionates will inhibit *Bacillus mesentericus*, the rope causing bacterium. Propionates are also used in a limited extent to inhibit mold growth in processed cheese.

The antifungal activity of propionic acid is weak compared to the other organic acids. Therefore, propionates must be used in relatively high concentrations to be effective. As with other organic acids, the pH value of the food to be preserved affects antimicrobial activity. Because of its low dissociation constant, propionic acid is active in a pH range similar to that of sorbic acid (Eckland 1990).

2.4 Medium-Chain Fatty Acids

Generally, fatty acids are most effective as inhibitors of gram-positive bacteria and yeasts, although some fatty acids exhibit antimycotic activity. Chipley et al. (1981) observed that fatty acid derivatives reduced growth and aflatoxin production by *Aspergillus* spp.

Polyhydric alcohol fatty acid esters have great potential for use as emulsifiers in food formulations (Razani-Rohani and Griffiths 1994). They also possess antifungal properties and, therefore, may exert a preservative effect in foods. Kato and Shibasaki (1975) demonstrated strong fungistatic activity of glycerol monopalmitate and glycerol monolaurate toward *Aspergillus niger*, *Penicillium citrinum*, *Candida utilis*, and *Saccharomyces cerevisiae*. Sucrose monopalmitate and sucrose monolaurate were found to be slightly inhibitory to a spoilage film-forming yeast inoculated into a soy sauce substrate (Kato 1981). Six sucrose esters substituted to different degrees with a mixture of palmitic and stearic acids were examined by

Marshall and Bullerman (1986) for antifungal properties. Growth of *Aspergillus*, *Penicillium*, *Cladosporium*, and *Alternaria* spp. were inhibited in media containing 1% of the sucrose esters.

3 ANTIBIOTICS

3.1 Natamycin

Natamycin, formerly called pimaricin is an antibiotic that possesses strong antifungal properties, yet is not active against bacteria. Its use is currently allowed in several countries. Researchers have demonstrated that natamycin is active at very low concentrations against many of the fungi known to cause food spoilage. Levels of 10 ppm have been reported to be effective in Swiss cheese to control the growth of *Penicillium citrinum* while solutions of 1000–2000 ppm are effective as dips for cheese (Jay 1982; Pugazhenth et al. 1999). Natamycin inhibits aflatoxin formation by molds only when growth was completely inhibited (Ray and Bullerman 1982).

3.2 Nisin

Nisin is active against gram-positive bacteria but has not been reported to be effective against yeasts and molds (Luchansky 1999).

4 METABOLITES FROM LACTIC ACID BACTERIA

A wide variety of raw foods are preserved by lactic acid fermentation, including milk, meat, fruits, and vegetables. Reduction of pH and removal of large amount of carbohydrates by fermentation are the primary preservation actions that lactic acid bacteria (LAB) provide to a fermented food. These actions are largely ineffective in preventing the growth of fungi in foods. However, it has also been recognized that LAB can produce inhibitory substances other than organic acid (acetate and acetate) that are antagonistic toward other microorganisms (Batish et al. 1997). The antibacterial properties of LAB are well documented. Several LAB, typically of the genera *Lactococcus* and *Lactobacillus* produce antibacterial substances. Antifungal properties of LAB have received little attention, however, several metabolites of LAB have been reported to have antifungal activity.

Batish et al. (1989) screened different lactic starter cultures for their antifungal activity with the goal of commercially exploiting their antifungal potentials. They found several strains of *Streptococcus* that inhibited a wide variety of molds. While the antifungal substances produced by the LAB were not identified or characterized, maximum production occurred at 30°C and pH 6.8. Several specific LAB metabolites have been reported to have antifungal activity.

4.1 Diacetyl

Diacetyl is a metabolic end product produced by some species of LAB. It is best known for the buttery aroma that it imparts to cultured dairy products. Its antimicrobial action has been investigated by Jay who reported that a concentration of 200 ppm was inhibitory to yeast and 300 ppm was inhibitory to molds (Jay 1992). Acidity of the growth medium was shown to have a direct effect on the antimicrobial activity of diacetyl. The compound was clearly more effective as an antifungal agent below 7.0 than above this value. Reasons for pH associated antifungal activity is not clear.

Since, effective concentration of diacetyl imparts a sharp odor of butter, potential for use in foods as an antifungal agent is limited. However, its use as a utensil sanitizer and in wash or rinse water for certain products is feasible.

4.2 Microgard

Microgard is grade A skim milk that has been fermented by *Propionibacterium shermanii* and then pasteurized (Hoover 2000). The product prolongs the shelf life of cottage cheese by inhibiting psychotropic spoilage bacteria (Lyon et al. 1993). The product is also antagonistic toward some yeast and molds. Microgard consists of proionic acid, diacetyl, acetic acid, and lactic acid (Al-Zoreky 1991).

4.3 Reuterin

Reuterin is a low molecular weight nonproteinaceous, highly soluble, pH neutral metabolite produced by *Lactobacillus reuterii*. The compound is a broad-spectrum antimicrobial with activity that encompasses yeast and molds as well as bacteria. It may have application in preservation of food by reducing populations of pathogenic and spoilage microorganisms (Daeshel and Penner 1992).

5 HERBS AND SPICES

Herbs and spices are widely used to impart flavor to foods. It is generally accepted that certain herbs and spices have antimicrobial activity and may influence the keeping quality of food to which they have been added. However, they are not currently used with the primary purpose of providing a preservative effect.

Hoffman and Evans (1911) were among the earliest to describe the preservative action of cinnamon, cloves, mustard, allspice, nutmeg, ginger, black pepper, and cayenne pepper. They found that cinnamon, cloves, and mustard were most effective and ginger, black pepper, and cayenne pepper were least effective.

Bachman (1982) studied the effect of spices and their essential oils on growth of several test organisms, including

Aspergillus and *Penicillium* species, and concluded that spices used in amounts as employed normally for ordinary food were insufficient as preservatives. However, when used in larger amount, cinnamon, cloves, and all spices retarded mold growth. Bullerman (1974) reported that cinnamon in concentrations as low as 0.02% inhibited mold growth and aflatoxin production in culture media and cinnamon bread.

Combinations of different levels of potassium sorbate with cloves showed enhanced or possibly synergistic inhibitory effect on the growth of molds, indicating the possibility of using spices and commercial antifungal agents together in small amount to obtain antifungal activity (Azzouz and Bullerman 1982).

In most instances, herbs and spices are not effective antifungal agents when used in amounts normally added to foods. However, when used in combination with other preservative systems, they can be valuable contributors to an antifungal system consisting of interacting physical and chemical preservatives (Naidiu 2000).

6 ESSENTIAL OILS

The antimicrobial activities of extracts from several types of plants and plant parts used as flavoring agents in foods and beverages have been recognized for many years. Some of these essential oils have antifungal properties. Conner and Beuchat (1984) documented the effects of garlic and onion against yeasts and other investigators have shown these extracts to be inhibitory to molds. Alderman and Marth (1976) examined the effects of lemon and orange oils on *Aspergillus flavus* and found when the citrus oils were added to grapefruit juice or glucose yeast extract medium at concentrations of 3000–3500 ppm, growth and aflatoxin production was suppressed. When orange oil was added to either medium at concentrations up to 7000 ppm, growth and aflatoxin production were greatly reduced although still evident. Recent publications have reported that the essential oils of anise, coriander, Roman chamomile, basil, and oregano were inhibitory to food and industrial yeasts (Chao et al. 2000; Elgayyar et al. 2001).

7 PHENOLIC ACIDS

Phenolic antioxidants have been shown by several researchers to possess antifungal activity. Chang and Branen (1975) demonstrated that in a glucose salt medium, 1000 ppm butylated hydroxyanisole (BHA) inhibited growth and aflatoxin production of *Aspergillus parasiticus* spores, and >250 ppm inhibited growth and aflatoxin production of *A. parasiticus* mycelia. However, they found that at 10 ppm of BHA, total aflatoxin production was more than twice that of the control, with virtually no effect on mycelial weight. These results indicate that at high levels, BHA may serve as an effective antifungal agent, however, at low levels BHA may actually stimulate aflatoxin production. The BHA has

been documented as inhibiting *A. flavus*, *A. parasiticus*, *Penicillium*, *Geotrichum*, *Byssoschlamys* species, and *S. cerevisiae* (Davidson and Naidu 2000).

Since, the primary use of these compounds in foods is as antioxidants, their effectiveness as antifungal agents in food systems has not been adequately studied. While results of experiments in growth media indicate that these compounds exert antifungal effects, extrapolation of these resulting to food systems should be done with caution. Interaction of these compounds with food components will undoubtedly affect their antifungal properties.

8 GASES AND MODIFIED ATMOSPHERES

Elimination of oxygen is often used as a control measure for inhibiting the growth of molds. Exclusion of oxygen will not prevent growth of yeasts. Studies on bakery products have demonstrated that atmospheric O_2 levels must be reduced to 0.1–1% to effectively inhibit growth of molds. In studies on toasted bread, Cerny (1979) demonstrated that visible mold would occur in 3 days in air; 5 days in 99% N_2 –1% O_2 ; and >100 days in 99.9% N_2 –0.1% O_2 , 99% CO_2 –1.0% O_2 , 99.8% CO_2 –0.2% O_2 , and 100% CO_2 . This study demonstrates that although molds are considered to be aerobic organisms, certain species have the ability to grow at very low levels of O_2 concentrations. Effectively controlling mold by simple gas flushing can be difficult in practice. Chemical oxygen scavenger can be used in place of or to supplement gas flushing (Farber 1991). Oxygen scavengers will also give protection against package leaks and infiltrations of O_2 through the package.

Carbon dioxide exerts antifungal action that supplements simple exclusion of O_2 and, thus is more effective than inert gas such as nitrogen. The gas probably exerts antifungal activity by altering intracellular pH levels (Gorris 1994).

Recent research has shown CO_2 to have potential use with food. Carbon monoxide inhibits yeast and molds that causes postharvest decay in fruits and vegetables (Wagner and Moberg 1989). The potential toxicity of this compound to workers requires special handling procedures.

Sulfur dioxide is broadly effective against yeasts and molds. It is used extensively to control growth of undesirable microorganism in fruits, fruit drinks, wines, sausages, fresh shrimp, and pickles. The antimicrobial activity of SO_2 is associated with the unionized form of the molecule. Therefore, it is most effective at pH values <4.0, where this form predominates (Weidzicha 2000).

Ethylene oxide has been widely used to reduce microbial contamination and to kill insects in various dried foods. The gas has been used to treat gums, spice, dried fruits, corn, wheat, barley, dried egg, and gelatin (ICMSF 1980). Concern over the toxicity of residues has limited the use of this gas in recent years.

Propylene oxide has been less studied than ethylene oxide. However, it appears that its antifungal effects are similar (Wagner and Moberg 1989). Yeasts and molds are more

sensitive to the gas than bacteria. Propylene oxide has been used as a fumigant for control of microorganisms and insects in bulk quantities of goods such as cocoa, gums, processed spice, starch, and processed nutmeats (ICMSF 1980).

Ozone (O_3) is a strong antimicrobial agent with numerous applications in the food industry. It has been used for decades in many countries and was recently given GRAS status in the United States. Ozone in the aqueous or gaseous phase is active against a wide range of bacteria, molds, and yeasts. Most applications are targeted to decontamination of fruit and vegetable surfaces by washing in ozonated water (Xu 1999). A second application is fruit and vegetable storage. Barth et al. (1995) assessed ozone exposure on storage of blackberries stored at 2°C in air with 0.3 ppm ozone. Fungal development was suppressed while 20% of the control fruits showed decay. The effectiveness of ozone is influenced by the intrinsic factors of a food. It also oxidizes food surfaces when used at high levels. Further research may reduce some of these concerns so ozone can be used in broader food applications.

9 INTERACTION OF FACTORS

Many of the antifungal agents reviewed in this chapter need to be used at extreme concentrations or levels in a food to be effective when used alone. However, a variety of factors can prevent growth of fungi. While fungi tend to be more tolerant to adverse environmental conditions than bacteria, combining inhibitory factors such as temperature, water activity, or pH with antifungal agents can result in considerable improvement of the microbial stability of foods. Suitable combinations of growth-limiting factors at subinhibitory levels can be devised so that certain microorganisms can no longer proliferate.

Sorbic acid at 1000 ppm and pH 7.0 will not inhibit mold growth. However, if the pH is lowered to 5.0, growth of most molds will be inhibited (Liewen and Marth 1985). Antioxidants such as BHA and BHT have been shown to potentiate the action of sorbic acid (Scott 1989). In general, antifungal food additives become more effective as environmental conditions move away from the optimum for a particular organism.

The level of a single growth-limiting factor that will inhibit a microorganism is usually determined under conditions in which all other factors are optimum. In preserving foods more than one factor is usually relied upon to control microbial growth. Addition of a substance, which in itself does not give full inhibition, can effectively preserve products in the presence of other subinhibitory factors. The effect of superimposing limiting factors is known as the “hurdles concept” (Leistner 1999).

Little information is currently available on combining subinhibitory factors to preserve food. It is very time consuming and expensive to design preservative systems using the hurdles concept by random design. Predicative modeling can be used to test the consequences of a number of factors changing at the same time. With proper design and

interpretation, preservative systems can be designed rapidly and efficiently (Whiting and Buchanan 1994). However, product challenge studies should be conducted to verify the effectiveness of a combination of subinhibitory factors (Labuza and Taoukis 1992).

10 CONCLUSIONS

Although antifungal food additives are, in general, the only successful way to control fungal growth in foods, they should never be used as a substitute for good manufacturing practices or proper sanitation procedures. Their proper use is as a processing aid. Obviously, antifungal food additive must be safe for human consumption, and their use is limited by law in most countries to relatively low levels and to specific foods. In addition, many of the traditional antifungal food additives are active only at high levels that adversely affect the taste of food and cannot be used commercially. However, with greater emphasis on the development and marketing of refrigerated foods by the food industry, some new preservation methods will become widely used and accepted.

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Molecular Detection of Fungi in Foods and Feeds

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1 INTRODUCTION

Spoilage is a serious problem for the food industry because it renders products unacceptable for consumption. Spoilage of foods and feeds is often the result of microbial activity from a variety of organisms. The microbial flora that will develop depends very much on both intrinsic and extrinsic parameters, modes of processing and preservation, and implicit parameters (Deak 1991; Van der Vossen and Hofstra 1996). Yeasts and moulds can be found in a variety of environments because they can utilize a variety of substrates, and are relatively tolerant to low pH, low water activity, low temperature and preservatives. Consequently, although only a limited range of fungal species are responsible for the spoilage of a given food (Filtenborg et al. 1996), contamination of foods and feeds by yeasts and moulds has been extensively reported (Huis in't Veld 1996). Fungi can contaminate foods and feeds at different stages including harvesting, processing and handling. Changes induced by spoilage of yeasts and moulds can be of a sensory nature, e.g., production of slime, pigmented growth, discoloration, rotting, development of off-odors and off-flavors. The most important aspect of food spoilage is, however, the formation of mycotoxins that may cause food poisonings. Although traditional morphological and physiological characters are fundamental parameters contributing to the identification of microorganisms in foods and feeds, these criteria may be influenced by environmental conditions. To supplement classical methods, a number of nucleic acid-based methods have been developed in the past few years. These methods have the advantage over phenotypic methods of not being influenced by environmental conditions of the cells since the nucleotide sequence of DNA remains constant during growth. This review focuses on molecular methods developed for direct detection of fungi in different foods and feeds. First, we

will discuss two basic types of detection methods, those based on DNA hybridization, and those based on DNA amplification. In the second part of the chapter, the possible nucleic acid targets of direct detection methods will be discussed.

2 METHODS USED FOR DETECTION OF FUNGI

2.1 Hybridization-Based Methods

In its basic application, DNA or RNA is fixed to a solid phase and a labeled probe is added and allowed to react with its complementary sequence (Southern 1975). The direct hybridization techniques used for the detection of fungi include *in situ* hybridization and colony or dot blot hybridization methods (Geisen 1998; Sterflinger et al. 1998). Besides DNA probes, peptide nucleic acid (PNA) probes have also been developed. Peptide nucleic acids are pseudopeptides in which the sugar phosphate backbone of DNA is replaced by a polyamide backbone. Due to the uncharged backbone, PNA probes have unique properties relative to oligonucleotides. These characteristics include faster hybridization kinetics, and the ability to form a stable PNA/NA hybrid even at low ion concentrations, necessary to disrupt the secondary structure of nucleic acids such as rRNA (Perry-O'Keefe et al. 2001a,b; Stender et al. 2001).

A recent development of hybridization-based techniques is the microarray technology. DNA microarrays are glass slides containing an ordered mosaic of the entire genome as a collection of either oligonucleotides (oligonucleotide microarrays) or PCR products representing individual genes (cDNA microarrays). The development of microarrays has been fuelled by the application of robotic technology to routine molecular biology. This technique allows a single hybridization to be performed against multiple replicates of a single

microbial genome, or against copies of several unrelated genomes on a single glass slide. Microarrays could be used to identify virulence genes as PCR products in food-borne bacteria (Chizhikov et al. 2001). Similarly, they could also be used for the direct detection of a variety of fungi contaminating foods and feeds.

Hybridization techniques have only rarely been used for direct detection of fungi in food matrices due to the lack of demanded sensitivity (Scheu et al. 1998). Kosse et al. (1997) developed 18 S rDNA-based probes for the detection of yogurt spoilage yeasts, while Geisen (1998) developed suitable probes for the detection of fumonisin producing *Fusarium* species based on randomly selected sequences. Chemiluminescent DNA probe kits are available for the detection of some medically important fungi including *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis*, and *Cryptococcus neoformans* (GenProbe, San Diego, USA). However, to our knowledge, such kits have not yet been developed for food-borne fungi.

2.2 Amplification-Based Methods

Sensitivity of detection can be greatly improved through the use of the different *in vitro* amplification methods [polymerase chain reaction (PCR), ligase chain reaction or self-sustained sequence replication, nucleic acid sequence-based amplification (NASBA)]. With the exception of PCR and NASBA, the above-mentioned *in vitro* amplification methods have had only limited practical relevance in food monitoring (e.g., Stubbs et al. 1994).

2.2.1 Polymerase Chain Reaction

Since its discovery, a considerable number of PCR-based assays have been developed, but they have been applied most often to clinical and environmental samples and more rarely for the detection of food-borne microorganisms. The PCR technique allows rapid and selective identification and/or detection of microorganisms in different matrices by amplifying specific gene fragments. The reaction cycle consists of three steps: (a) denaturation of the double-stranded DNA (dsDNA), (b) annealing of short DNA fragments (primers) to single DNA strands, and (c) extension of the primers with a thermostable DNA polymerase. Following the completion of one cycle, the sample is denatured for the next annealing and extension steps during which not only the original target region is amplified, but also the amplification product of the first cycle, leading to an exponential increase of the number of copies of the target DNA. The detection of amplification products is possible through gel electrophoresis, ethidium bromide staining and visual examination of the gel using ultraviolet light. To increase the sensitivity and to confirm the identity of the amplification product, Southern blotting and hybridization with a specific probe can also be carried out (Hendolin et al. 2000; Löffler et al. 2000; Sandhu et al. 1995). Attempts have been made to increase the

specificity of PCR reactions using other methods, including post-PCR hybridization (Sandhu et al. 1995), PCR-ELISA (enzyme linked immunosorbent assay) or ELOSA (enzyme linked oligosorbent assay) reactions (Grimm and Geisen 1998; Schnerr et al. 2001), RFLP (restriction fragment length polymorphism) analysis of the PCR products (Yamagishi et al. 1999), denaturing gradient gel electrophoresis (DGGE, Cocolin et al. 2001), fluorescent capillary electrophoresis (Turenne et al. 1999), or nested PCR, where one set of primers is used to amplify DNA fragments from target DNA, and a second set of primers complementary to an internal sequence of the product of the first PCR reaction is used to score and confirm the results (Ibeas 1997). In case of closely related species, single nucleotide differences can be visualized by using single strand conformation polymorphism (SSCP) analysis (Kumeda and Asao 1996), heteroduplex mobility assay (Olicio et al. 1999), heteroduplex panel analysis (Kumeda and Asao 2001), or by sequence analysis (Cappa and Cocconcelli 2001).

2.2.2 Nucleic Acid Sequence-Based Amplification

An alternative to PCR analysis, NASBA has also been applied to detect fungi (Löffler et al. 2001; Widjoatmodjo et al. 1999). The NASBA is an isothermal nucleic acid amplification technology that specifically amplifies RNA sequences using T7 RNA polymerase (Compton 1991). Major advantages of NASBA over PCR is that it is performed isothermally at 41°C, no separate reverse transcription step is required for RNA amplification, and since RNA is less stable than DNA—provides a better estimate of living cells in the sample analyzed.

2.2.3 Quantification of Results

Besides qualitative detection of spoilage microbes, it is also often desirable to know their abundance in foods and feeds. Quantification of the PCR results can be carried out by different approaches including limiting dilution of the DNA samples, densitometric measurement of PCR products, by HPLC with a UV detector (Katz 1996), by quantitative competitive PCR (QC-PCR), or by real-time PCR (Cross 1995). Two of them, QC-PCR and real-time PCR are the most promising tools for quantifying fungi in foods and feeds.

The QC-PCR using internal DNA standard provide the means for determining relative amounts of target DNA. The principle of QC-PCR is the coamplification of standard DNA together with target DNA. The competitor DNA is of known sequence (typically identical with the target DNA with added deletions or insertions) and has the same primer binding sites as the target DNA (Baek and Kenerley 1998; Haugland et al. 1999).

During conventional PCR, an endpoint analysis is carried out by examining the fluorescence of ethidium bromide stained amplification products separated by gel electrophoresis. With real-time PCR, the continuous analysis of amplification-associated fluorescence during the whole PCR

reaction gives a graphic display of the time course of amplification of the PCR product of interest. Both direct and indirect methods are used for generating the fluorescence monitored during the PCR cycle (Walker 2001 and references therein; Figure 1). Indirect assays employ a system that yields fluorescence generated during the process of primer extension during amplification. This is employed in the Taqman system. Taqman probes utilize the intrinsic 5' nuclease activity of Taq DNA polymerase to digest a probe that has annealed to the specific gene of interest. The probe consists of quencher and reporter fluorochromes separated by a specific DNA sequence. Taq DNA polymerase-associated 5' nuclease digestion of the probe results in degradation of the probe and separation of the fluorochromes, so loss of quenching results in amplification-associated production of fluorescence. Direct methods refer to those systems in which fluorescence is a direct result of some binding of a fluorescence molecule to the amplified product or a direct incorporation of a fluorescence interference probe into the amplified product. A simple direct method involves the use of a fluorescent dye such as SYBR Green. This dye possesses selective affinity to dsDNA. Binding of the dye to dsDNA enhances fluorescence at 530 nm proportionally with dsDNA concentration. Specific hybridization probes can also be used that fluoresce only when bound to the gene of interest. All these probes are based on fluorescent resonance energy transfer (FRET; Walker 2001). Molecular beacons are essentially hairpin probes that employ fluorescence interference (Figure 1). A variation on

the hairpin probe concept has been employed to develop unimolecular probe systems such as the Sunset or Scorpion primers, which are incorporated into the amplified sequence during the PCR reaction. A variety of PNA-based probes have also been developed for real-time PCR applications (Stender et al. 2002).

The LightCycler hybridization probe system consists of a labeled donor and acceptor probe. Fluorescence from the acceptor will only be generated when both probes are annealed to the product (Figure 1). The level of fluorescence is proportional to the amount of DNA generated during the PCR reaction (Löffler et al. 2000). The PCR reaction can be carried out in small volumes in a glass capillary that speeds up the heating process. The LightCycler technology combines rapid *in vitro* amplification in glass capillaries with real-time determination and quantification of DNA, enabling a 35 cycle PCR with 32 samples to be completed in 45 min.

2.2.4 Limits of PCR

One of the main drawbacks of PCR-based detection methods is that besides living cells, dead cells with relatively intact DNA are also detected, thus leading to false positive results. This limitation can be circumvented by including a propagation step prior to PCR analysis, or by using RNA as template in reverse transcription coupled with PCR, or NASBA reactions (Vaitilingom et al. 1998; Widjoatmodjo et al. 1999). Although the detection of dead cells is a

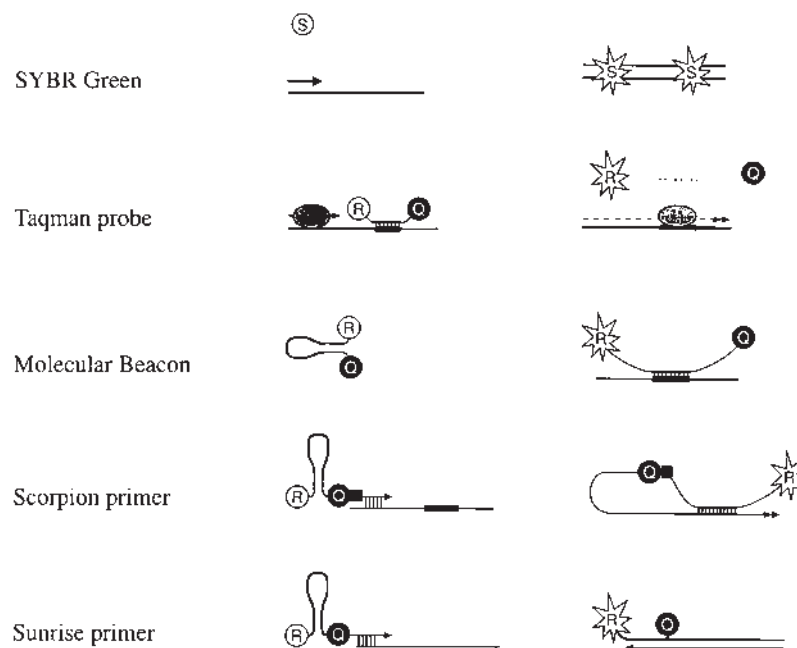


Figure 1 Fluorescence systems used in real-time PCR systems. S, SYBR Green; R, reporter fluorochrome; Q, quencher fluorochrome. Separation of the quencher from proximity to the reporter enables the fluorescence of the reporter to be measured [from Walker (2001); reprinted by permission of John Wiley & Sons, Inc.].

disadvantage for spoilage bacteria and yeasts, Geisen (1998) suggested that it is advantageous in the case of mycotoxin-producing fungi. Mycotoxins are stable molecules, and detection of the producing fungi in a sample could be used as prediction for the presence of mycotoxins.

Much of the difficulty in implementing PCR for the analysis of food samples lies in the problems encountered during the preparation of template DNAs from food matrices. The DNA extraction method used must achieve two aims: efficiently purify fungal DNA with minimal loss, and remove compounds inhibiting the PCR reaction. Foods are complex matrices and may contain several compounds that interfere with the PCR reaction leading to false negative results. These compounds include nucleases, chelating agents, or inhibitors of the polymerase itself. Cheese was found to be an extremely problematic matrix (Scheu et al. 1998). Several attempts have been made to develop a DNA extraction protocol to remove inhibitors from the samples (Dickinson et al. 1995; Lantz et al. 1999; Rossen et al. 1991; Rossen et al. 1992). The use of additional reagents during DNA extraction was suggested by different authors (e.g., DNA binding agents such as hexadecyl-trimethylammonium bromide, or polyvinyl pyrrolidone for elimination of polyphenols). Additionally, DNA samples can be further purified by dialysis, gel filtration, or by other chromatographic methods. Besides careful extraction, other methods have also been suggested, including extensive dilution of the contaminating substances, or application of internal standard DNA in the PCR reaction (Geisen 1998). Separation of the microbes from the food matrix can be carried out by subculturing, or by using magnetic beads coated with specific binding proteins (lectins, antibodies; Patel et al. 1993).

3 MOLECULAR TARGETS USED FOR THE DETECTION OF FUNGI

The choice of molecular target depends on the aim wished to be achieved. For panfungal detection, usually the conserved

regions the rRNA gene cluster are targeted. Other targets could also be used, including genes of the ergosterol biosynthesis (Morace et al. 1997), translation elongation factor genes (Vaitilingom et al. 1998), and the chitin synthase gene (Jordan 1994). For specific detection of a single genus or species, more variable regions of the genome, e.g., spacer regions of the rRNA gene cluster, or sequence characterized amplified region (SCAR) markers should be targeted. For the detection of mycotoxin producing fungi, sequences of the mycotoxin biosynthetic genes are the best targets. In the following, the targets used for molecular detection of fungi are dealt with, with special emphasis on mycotoxin producing fungi.

3.1 Targets Based on the Ribosomal RNA Gene Cluster

The ribosomal RNA gene cluster occurs in fungi as tandem repeats of a structured unit comprising three ribosomal RNA subunit genes, internal transcribed spacers (ITS) and intergenic spacers (IGS) (Figure 2). The DNA sequences within the subunits in general contain some extremely conserved sequences useful for the development of broad-spectrum primers that allow amplification of fungal sequences from mixed DNA samples. The resulting fragments can be further fractionated to obtain species or genus specific profiles by other techniques, e.g., nested PCR, SSCP, hybridization, ELOSA, RFLP analysis, or sequencing. The more variable spacer regions can be used for genus- or species-specific detection approaches.

3.1.1 18S rRNA Gene As Target

There are several reports where 18S rDNA sequences have been used for the detection and identification of fungi. Kappe et al. (1996), Smit et al. (1999), and Borneman and Hartin (2000) developed primer pairs based on this region for the

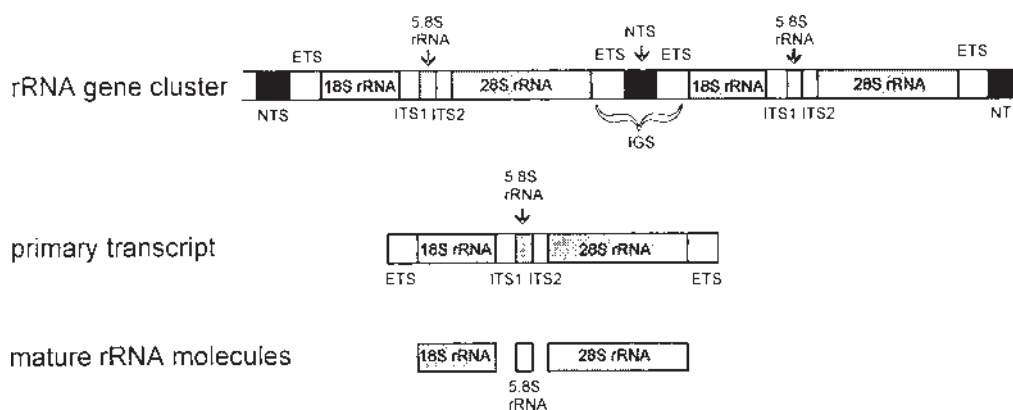


Figure 2 Organization of the ribosomal RNA gene cluster in filamentous fungi. ETS, external transcribed spacer; NTS, nontranscribed spacer; IGS, intergenic spacer; ITS1 and ITS2, internal transcribed spacer regions.

detection of a wide range of fungi. Mayer (2002) also developed a primer pair and a Taqman probe based on 18 S rDNA sequences, which was used successfully for real-time PCR detection of fungi in black pepper, red pepper, corn, and cereal samples. Makimura et al. (1994) developed a PCR detection system based on 18S rDNA sequences for detection of *Aspergillus* and *Penicillium* species. Cappa and Cocconcelli (2001) developed an 18 S rRNA-based assay for the detection of fungi in food samples.

3.1.2 28 S rRNA Gene As Target

Although the DNA coding for the large (28S) ribosomal rRNA subunit is relatively conserved and is more commonly used for work at higher taxonomic levels, certain portions of it, particularly the eukaryotic D1 and D2 divergent domains near the 5' end are variable enough to detect species-specific differences. The D1–D2 region has extensively been used for phylogenetic studies of aspergilli, penicillia, and yeasts as well as other fungi (Peterson 2000; Rigó et al. 2001). Universal 28S rDNA-based primers were developed by Sandhu et al. (1995). These authors used species-specific probes in post-PCR hybridization reactions to detect the presence of fungi of interest even in situations containing mixed fungal species.

3.1.3 The ITS Region As Target

The ITS region has been most frequently used as target for species-specific detection of fungi in foods and feeds. This region was targeted for the detection of spoilage yeasts including *Zygosaccharomyces* sp. and *Torulaspora delbrueckii* (Sancho et al. 2000), *Saccharomyces cerevisiae* (Arlorio et al. 1999), *Alternaria* sp. (Zur et al. 1999), *Penicillia* (Pedersen et al. 1997; Boysen et al. 2000), and *Fusarium avenaceum* (Schilling 1995). Olsson (2000) applied a QC-PCR approach using ITS-based primers for the detection of *Penicillium* species including *Penicillium roquefortii* in cereals. Grimm and Geisen (1998) developed ITS-based primer pairs for the detection of fumonisin producing *Fusarium* species. The sensitivity of the assay was increased using PCR-ELISA. Hendolin et al. (2000) developed a panfungal PCR technique coupled with multiplex liquid hybridization based on ITS specific primers for the detection of a number of fungi in clinical specimens.

3.1.4 Other rDNA Regions Used As Targets

Although the ITS region allows for discrimination of closely-related species, it may not be sufficiently variable to distinguish sibling biological species or isolates. The intergenic spacer region of the nuclear rDNA (IGS) however, has been used for species determination when ITS regions lacked sufficient variation (Spreadbury et al. 1993). Ribosomal RNAs are also encoded in the mitochondria and have been used for molecular identification (Wakefield et al. 1990). Fulton and Brown (1997) developed a primer pair

based on sequences of an intron located within the 18 S rRNA gene for the detection of *Monilinia fructicola*. Montone and Litzky (1995) targeted the 5 S rRNA gene for the detection of different *Aspergillus* species.

3.2 Mycotoxin Biosynthetic Genes As Targets for Detection of Fungi

Mycotoxins are a chemically diverse group of fungal secondary metabolites that are harmful to animals and humans. Several hundreds of different mycotoxins have been identified, but only about 20 of them are relevant to human health. Most of the mycotoxin-producing species are filamentous ascomycetes or deuteromycetes, *Aspergillus*, *Fusarium* and *Penicillium* are considered as being the most important mycotoxin-producing genera. Most mycotoxins are very resistant to physical or chemical treatments. Although well-documented cases of mycotoxicoses are rare, the constant uptake of small amounts of mycotoxins, especially those with carcinogenic activity, can have profound effects on human health. Apart from the use of specific rDNA gene-based or RAPD-based probes, mycotoxin biosynthetic genes can serve as ideal targets for the detection of the producing fungi. To date, a number of biosynthetic genes of mycotoxins have been isolated and characterized. These gene sequences could serve as targets for molecular detection of the producing fungi as detailed below.

3.2.1 Aflatoxins

Aflatoxins (ATs) are among the most carcinogenic naturally occurring compounds known. Aflatoxins are produced mainly by species of *Aspergillus* section *Flavi*, e.g., by *A. flavus*, *A. parasiticus*, *A. nomius*, and *A. bombycis*. Sterigmatocystin, an intermediate of AT biosynthesis is also produced by other fungal species including, e.g., *Aspergillus nidulans* and *A. versicolor*. *A. flavus* and *A. parasiticus* are closely related to the nonaflatoxigenic *A. oryzae* and *A. sojae* species, respectively, which are used in food industry for producing soy sauce and frequently applied as hosts for the expression of heterologous proteins. Since these species are both morphologically and physiologically very similar to the AT-producing species, molecular methods have been extensively surveyed for the differentiation of these species, and AT-producing and nonproducing *A. flavus* isolates.

The biosynthetic pathway leading to AT production is one of the best known secondary metabolite pathways in fungi (Brown et al. 1996; 1999). Criseo et al. (2001), Färber et al. (1997), Geisen (1996; 1998), and Shapira et al. (1996) applied multiplex PCR targeting 3–4 genes of the gene cluster to identify AT-producing fungi. Bagnara et al. (2000) applied the real-time PCR system for the detection of an AT producing *A. flavus* isolate in black pepper. Mayer et al. (2003) developed a Taqman probe based on sequences of the norsolorinic acid reductase (*nor-1*) gene for quantitative detection of AT-producing fungi in foods including black

pepper, red pepper, corn, and cereals. All authors found that some nonaflatoxigenic isolates gave false positive results. Even the AT nonproducing *A. sojae* and *A. oryzae* isolates were found to carry AT biosynthetic genes (Kusumoto et al. 2000; Matsushima et al. 2001). However, mRNA of the *aflR*, the regulatory gene of AT biosynthesis was not observed in *A. sojae* (Matsushima et al. 2001). These results indicate that a (multiplex) RT-PCR technique developed by Sweeney et al. (2000) for monitoring AT production in *A. parasiticus* could be used more efficiently for detection of AT producing fungi. Mayer et al. (2001) used Taqman-based real-time PCR for monitoring the expression of the *nor-1* gene providing a possible mRNA-based method for the detection of AT-producing fungi in foods and feeds.

3.2.2 Trichothecenes

Trichothecenes are sesquiterpenoid mycotoxins produced by several fungal genera including *Fusarium*, *Trichothecium*, *Acremonium*, *Gliocladium*, *Myrothecium*, *Trichoderma* and *Stachybotrys*. More than 30 structurally related tetracyclic trichothecenes are known, among which T-2 toxin, diacetoxyscirpenol, deoxynivalenol, and nivalenol are economically the most important mycotoxins. The main producers of these toxins are in the genus *Fusarium* (e.g., *F. acuminatum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. solani*, and *F. sporotrichioides*). Trichothecenes have dermatotoxic, cytotoxic, and phytotoxic properties, and have been suggested to play a role in plant pathogenesis as virulence factors. Trichothecene biosynthesis genes were found to comprise a 25 kbp gene cluster including at least 10 genes in *F. sporotrichioides* and *F. graminearum* (Brown et al. 2001; Hohn et al. 1993). Similar clustering of the trichothecene biosynthesis genes was found in *Myrothecium roridum*, which produces macrocyclic trichothecenes (Trapp et al. 1998).

Fekete et al. (1997) determined the sequence of the *Tri5* gene of *Fusarium poae*, and used a 378 bp fragment of it as a hybridization probe to detect similar sequences by dot blot hybridization in other fungi. Strong hybridization was observed to trichothecene producing *Fusarium*, *Myrothecium* and *Stachybotrys* species, but not to *Trichoderma* and *Trichothecium* DNAs. Niessen and Vogel (1998) developed a PCR method based on the amplification of *Tri5* sequences for the detection of trichothecene producing *Fusarium* species in wheat samples. The authors also applied the LightCycler system with Sybr Green I for quantitation of their results. For distinguishing nonspecific products such as primer dimers, melting point analysis was carried out (Schnerr et al. 2001). *Tri5* gene sequences were chosen as targets too in a quantitative competitive PCR approach developed for the detection and quantification of trichothecene producing fusaria in cereals by Edwards et al. (2001), and by Birzele et al. (2000). Doohan et al. (1999) developed a reverse-transcription-based PCR assay to quantify the expression of the *Tri5* gene in *Fusarium* species. Lee et al. (2001) developed primer pairs based on sequences of the *Tri7*

gene for distinguishing between deoxynivalenol and nivalenol producing isolates of *Gibberella zeae* (*Fusarium graminearum*).

3.2.3 Patulin

Patulin is an unsaturated lactone produced by a number of *Aspergillus*, *Penicillium*, and *Byssoschlamys* (*Paecilomyces*) species. Representatives of other fungal genera, such as *Mucor*, *Mortierella*, *Alternaria*, *Chrysosporium*, *Fusarium*, and *Trichoderma* were also found to produce this mycotoxin (Steiman et al. 1989). The economically most important producer of patulin is *Penicillium expansum*, the causative agent of soft rot of apples and other pomaceous fruits. Patulin is receiving worldwide attention because of its occurrence in unfermented apple juice. The biosynthesis of patulin is well known (Paterson et al. 2000). Two genes of the biosynthetic pathway, the polyketide synthase gene, and an iso-epoxydon dehydrogenase (IDH) gene have been cloned and characterized to date (Beck et al. 1990; Wang et al. 1991; Gaucher GM, and Fedeschko RW, unpublished results). The IDH gene product catalyzes the epoxydon-phyllostine oxidation step of patulin biosynthesis (Sekiguchi and Gaucher 1979). Recently, a primer pair specific for the IDH gene (GenBank accession number AF006680) has been developed and used successfully to detect patulin-producing abilities of penicillia (Paterson et al. 2000). Patulin production of *Aspergillus* species was also analyzed using analytical procedures (thin layer chromatography and HPLC), agar diffusion test, and a PCR-based approach using the primer pair developed for the detection of the IDH gene (Varga et al. 2003). The analytical-, biological-, and PCR-based approaches used for patulin detection gave highly similar results indicating that the primers developed for a *Penicillium* IDH gene could also be used to detect patulin producing aspergilli in natural substrates. A quantitative PCR approach is also being tested for the detection of patulin producing organisms in foods including apple products and malt.

3.2.4 Other Mycotoxin Biosynthetic Genes

The PR-toxin is a sesquiterpenoid mycotoxin produced by *P. roqueforti* strains isolated from cheese and silage (Geisen 1998). This mycotoxin has been implicated in incidences of mycotoxicoses resulting from the consumption of contaminated grains (Proctor and Hohn 1993). The gene sequence of aristolochene synthase (a sesquiterpene cyclase), the key enzyme of PR-toxin biosynthesis is known (Proctor and Hohn 1993). Geisen (1998) developed a primer pair based on the sequence of aristolochene synthase, which was used successfully for the detection of PR-toxin producing fungi in cereals and cheese.

The ergot alkaloids are produced mainly by *Claviceps purpurea* and some other *Claviceps* species (e.g., *C. paspali*, *C. fusiformis*), although other species, e.g., *Aspergillus fumigatus*, *A. clavatus*, *Penicillium* species, and plants have also been described as producers of these mycotoxins. Since

Claviceps strains parasitize not only cereals but also different kinds of grasses, ergot poisoning of grazing animals is still an economic problem. Sequences of some of the biosynthetic genes responsible for ergot alkaloid production are known (Tudzynski et al. 1999). Boichenko et al. (2001) developed a primer pair based on sequences of 4-(γ,γ -dimethylallyl)-tryptophane (DMAT) synthase, the key enzyme of the ergot alkaloid biosynthesis, and used successfully in PCR reactions to detect ergot alkaloid producing fungi.

Fumonisin B₁ was discovered in 1988 in *Fusarium moniliforme* (*F. verticillioides*) isolates. Fumonisin is most frequently encountered from stored corn and other cereals, and are the causal agents of leucoencephalomalacia, a fatal brain disease of horses, pulmonary edema in pigs and suspected to be responsible for high incidences of esophageal cancer in South Africa and China. Fumonisin is synthesized through the polyketide biosynthetic route. Seo et al. (2001) identified four coregulated genes associated with fumonisin production in *F. verticillioides*. These data indicate that these gene sequences could be used for the development of gene probes for the identification of potential fumonisin producing species in foods and feed products.

Since biosynthetic genes of other mycotoxins, e.g., those of fumonisins (Proctor et al. 1999; Seo et al. 2001) and the tremorgenic mycotoxin paxilline (Young et al. 2001) have also been characterized, molecular detection methods can potentially be applied in these cases as well. Apart from the mycotoxins mentioned earlier, the occurrence of the nephrotoxic ochratoxins in cereals, coffee beans, and other agricultural commodities also poses serious hazard to human and animal health (Varga et al. 2001a,b). Experiments are in progress in some European laboratories to isolate and characterize genes responsible for ochratoxin biosynthesis in *Penicillium* and *Aspergillus* species, and to identify suitable molecular probes for the detection of ochratoxin-producing fungi in foods and feeds (Quality of life and management of living resources project number QLK1-CT-1999-00433: Prevention of ochratoxin A in cereals; QLK1-CT-1999-01380: Early detection of toxigenic *Fusarium* species and ochratoxigenic fungi in plant products).

3.3 Other Sequences As Targets

One approach used for developing suitable species- or strain-specific probes for the detection of fungi is based on the random amplified polymorphic DNA technique (RAPD) (Williams et al. 1990). The RAPD is a variation of conventional PCR where one primer of arbitrary sequence is used, and the annealing temperature is low (usually 35°C). Species- or strain-specific RAPD fragments are selected, sequenced, and suitable primers are devised to amplify the specific fragment in conventional PCR reactions. Such SCAR markers have been successfully used to develop species-specific probes for a number of *Fusarium* species (Chelkowski et al. 1999; Nicholson et al. 1996; Nicholson et al. 1998; Schilling et al. 1996; Young et al. 2001),

particularly fumonisin-producing fusaria (Geisen 1998), and for *Aspergillus fumigatus* (Brandt et al. 1998; Varga et al. 2002, unpublished results). Murillo et al. (1998) developed a primer pair based on the sequence of a random genomic clone for the detection of *Fusarium moniliforme*.

For the detection of *Fusarium graminearum*, another target, the galactose oxidase (*gaoA*) gene has been used (Niessen and Vogel 1997). This enzyme is produced by only a limited number of fungi including *F. graminearum*, *Gibberella fujikuroi*, and *Beltraniella portoricensis*. Niessen et al. (1998) also developed a solid phase PCR with detection of immobilized amplified product in a one-phase system (DIAPOPS) to specifically amplify and quantify a DNA fragment of the *gaoA* gene from *F. graminearum*.

Microsatellite-based probes were developed for the detection of *Epichloë* species in different grasses (Groppe and Boller 1997). Mayer (2002) developed a *Penicillium nordicum* specific primer pair and a Taqman probe based on a polyketide synthase gene sequence for the detection of this species in cereal samples. Pearson and McKee (1992) developed a multiplex PCR method based on plasmid sequences for the detection and discrimination of *S. cerevisiae*, *Zygosaccharomyces bailii*, and *Z. rouxii* in foods. Pearson et al. (1995) designed a PCR-based assay to detect retrotransposon long terminal repeat elements for the detection of *Pichia membranefaciens*.

4 CONCLUSIONS

Fungi responsible for food spoilage cause significant economic losses for food manufacturers. In order to minimize food spoilage and be able to predict the quality and shelf-life of a particular food, a better understanding of the mechanisms underlying food spoilage is essential. To achieve this goal, there is a need for the development of direct detection methods for spoilage organisms including fungi. The main aim being to develop a rapid, sensitive, robust, and specific method to use directly on the sample for the detection of food spoilage organisms. Rapid identification of spoilage organisms is of profound importance to the food industry. This will enable intervention with appropriate measures to prevent serious economic losses. Rapid molecular techniques are valuable tools for screening foods and feeds for fungi. Among these, hybridization techniques have only rarely been used for direct detection of fungi, although novel developments in this field, including the application of PNA probes, and microarray techniques are encouraging. Amplification-based methods are frequently applied for direct detection of spoilage organisms. Most PCR-based detection methods target part of the ribosomal RNA gene cluster, although SCAR markers are also widely used. For the detection of mycotoxin producing fungi, the most direct procedure is targeting of the mycotoxin biosynthetic genes. Such approaches have been used recently for the detection of a number of fungi-producing mycotoxins such as ATs, patulin, PR toxin, and trichothecenes, and projects for the detection of

fungi producing ochratoxins and fumonisins are in progress. The recent development of real-time PCR methodology has made it possible to quantify the amount of organisms in foods and feeds.

Sensitivity of a molecular method depends not only on the detection system, but also to a great extent on the food matrix. In order to increase sensitivity, adequate protocols have to be established in order to discern potential PCR inhibitors. The speed, accuracy, and reliability of detection methods can greatly be enhanced by enrichment procedures including the use of magnetic particles coated with specific binding proteins (antibodies or lectins); by optimizing the DNA extraction protocol; or by using internal standard DNA in the PCR reaction. One of the main drawbacks of PCR-based detection methods is that DNA from dead cells can also be amplified leading to false positive results. This limitation can be addressed by including a propagation step prior to PCR, by using mRNA as template in NASBA or RT-PCR reactions. On the other hand, this disadvantage is only relative, since detection of dead mycotoxin producing fungi in a food matrix can serve as a prediction for mycotoxin contamination of the food.

The detection of microorganisms in food is still a time-consuming method particularly in the case of large-scale testing. The increasing availability of molecular kits for the detection of food-borne pathogenic bacteria is a step toward standardization of the molecular techniques. The development of similar kits for fungi causing food spoilage would be a further necessary step for the authorization of molecular methods as accepted detection methods in food microbiology.

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The Role of Spoilage Fungi in Seed Deterioration

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1 INTRODUCTION

Microorganisms are ubiquitously present in terrestrial ecosystems from which they are disseminated and contaminate plant communities. The ripening seed is no exception and is contaminated by a wide range of bacteria, yeasts, and filamentous fungi via the air, insects, rain splash, equipment, and agronomic practices. When seeds/cereal grains are harvested they thus carry a wide range of microbial contaminants. Postharvest treatment of such seeds and the prevailing environmental factors are key determinants of the impact that they may have on the quality of the seeds, including germinability. It is important to remember that both the harvested seeds/grains and contaminating microorganisms are alive and respiring slowly under dry, safe storage conditions.

Poor postharvest management can lead to rapid deterioration in quality characteristics and severely decrease germinability and nutritional value of the seeds. Microbial activity can cause undesirable effects in grains including discoloration, contribute to heating and losses in dry matter through the utilization of carbohydrates as energy sources, degrade lipids and proteins or alter their digestibility, produce volatile metabolites giving off-odors, cause loss of germination and baking and malting quality, affect use as animal feed or as seed, and filamentous fungal spoilage organisms may also produce mycotoxins that are highly toxic or carcinogenic or cause feed refusal and emesis (Christensen 1973). The spores of some fungi can also cause respiratory disease hazards to exposed workers (Lacey and Crook 1988).

Estimated losses of seeds, especially staple cereal grains in store from all causes varies widely. They may amount to 10% worldwide (Anon 1979) but can reach 50% in tropical regions (Hall 1970). Vassan (1980) estimated losses of high moisture

paddy in southern India exceeded 15–25% in only 9 days, while Rohani et al. (1985) found storage losses of paddy in West Malaysia of only 1%.

Deterioration of grain by microorganisms is determined by several factors which can be classified into four main groups: intrinsic factors (those which depend on the characteristic of the growth substrate), extrinsic factors (those imposed from the outside), processing factors (those resulting from the agronomic practices and food processing, which primarily modify the composition of the microflora), and implicit factors (those depending on the particular dominant microbial flora that initially develop in response to the intrinsic, processing, and extrinsic factors) (Sinha 1995). Figure 1 summarizes the factors affecting fungal colonization of the grain.

Wallace and Sinha (1981) in the 1970s were the first to consider stored seeds as a man-made ecosystem which needed to be examined in a more holistic and ecological manner to enable a proper understanding of the processes occurring and to improve postharvest management of stored seeds of all types. This in many respects enabled prevention strategies to be developed and implemented to avoid microbial and pest infestation from damaging seeds. Generally, since most seeds are stored dry, bacteria seldom cause biodeterioration problems. At intermediate moisture content levels fungal spoilage and pests are of major concern. This Chapter will endeavor to examine some of these important abiotic and biotic factors and their interactions that determine whether deterioration will occur and the dominant fungal species which may be involved. This is important as the fungal community structure influences the type of deterioration and whether mycotoxins are produced. Cereal grains particularly wheat, barley, maize, and rice are used as examples of seed systems, since they represent the key staple seeds worldwide.

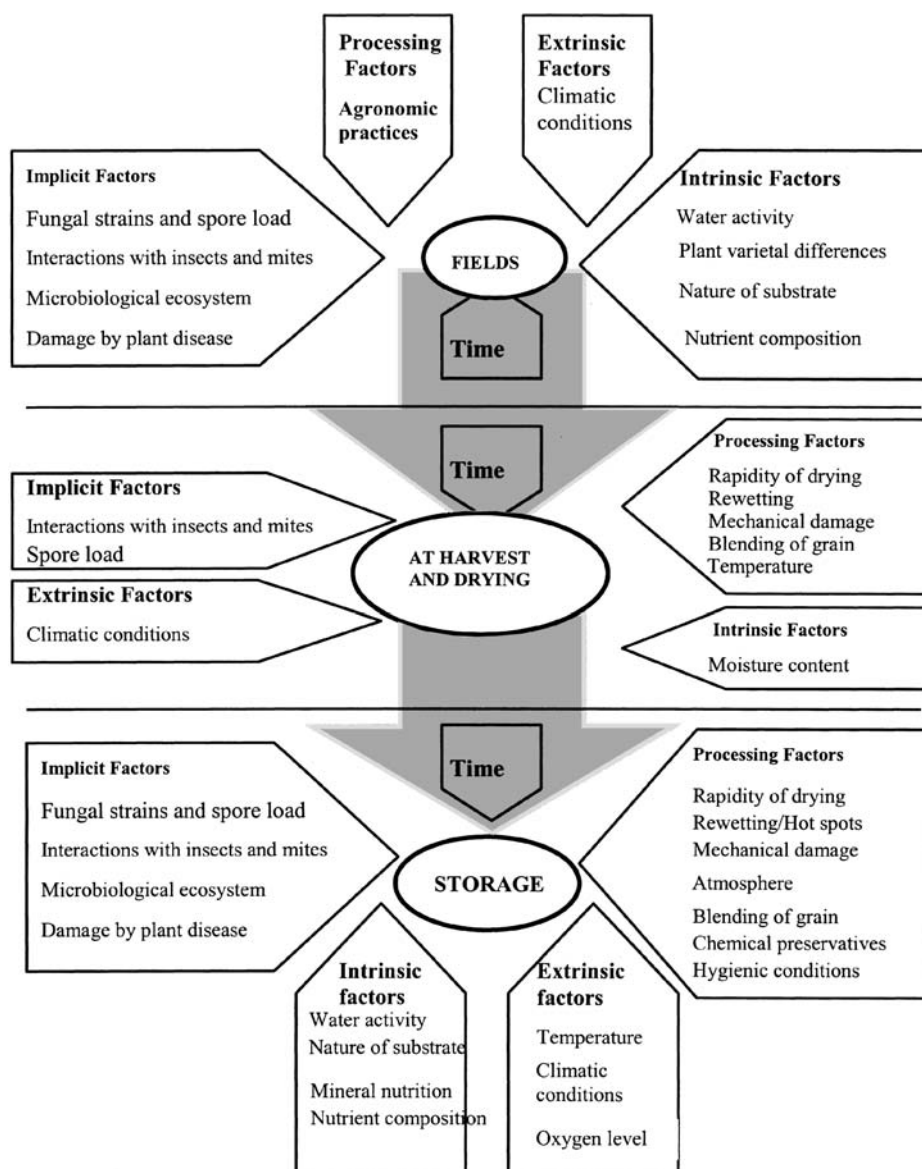


Figure 1 Integration of the most important abiotic and biotic factors impacting on grain spoilage.

2 ENVIRONMENTAL FACTORS WHICH INFLUENCE FUNGAL ACTIVITY IN THE STORED SEED ECOSYSTEM

2.1 Water Availability—The Concept

2.1.1 Fundamental Principles

The extent to which seeds and grain are susceptible to spoilage fungi depends, above all, on the presence of water in the system. In fact, many authors have written about the fundamental requirement for water in microbial growth

(Cooke and Whipps 1993; Griffin 1981; Scott 1957). Most stored products such as seeds and grain are hygroscopic in nature, i.e., they will either take up or release moisture until they reach a dynamic equilibrium with the surrounding environment. It is straightforward to express this water in terms of the percentage moisture content, based on the ratio of the dry weight to the wet weight (expressed either on a wet weight or dry weight basis). This, however, implies that the stored material consists of dry matter plus a certain amount of “attached” liquid water. In fact the situation is more complex than this. This is principally because the water in these substrates is not all equivalent, but held in a number of

different “states.” Broadly speaking, three main states of water can be identified in a hygroscopic material: (a) constitutive water; water that is chemically linked to the substrate material and forms part of its structural makeup, (b) adsorbed water, which is closely linked with the substrate surface by physical interactions, and (c) absorbed water, which is loosely associated with the substrate surface (Pixton 1967). In practice, probably there is no clear demarcation between these different states; the relative proportions of each type will depend on the overall moisture content of the material. The absorbed water fraction will become more weakly bound as further layers of water molecules accumulate on the surfaces (i.e., as the substrate becomes wetter). It is the loosely held water that provides the “free” or “available” water, since it is the fraction that is most readily available for microbial growth, although its overall availability will be influenced by the presence of solutes. The important implication here is that microorganisms, including spoilage fungi, respond not to water content but to water *availability*. Therefore, the concept of water availability is of fundamental importance in relation to microbial growth and spoilage.

2.1.2 Water Activity

Water availability itself can be expressed in a number of ways, one of the most convenient being water activity (a_w). The free water in a system is the proportion of the total water that is immediately available to reach equilibrium with the surrounding atmosphere. This will be reflected in the equilibrium vapor pressure or equilibrium relative humidity (ERH) exerted by the system. Water activity is defined as the ratio of the vapor pressure of the water in a substrate to that of pure water at the same temperature and pressure (Scott 1957). It is, therefore, directly linked to the ERH by the expression:

$$a_w = p/p_0 = \text{ERH}(\%)/100 \quad (\text{Labuza 1974})$$

where p is the vapor pressure of water in solution or solid substrate, p_0 , vapor pressure of pure water at experimental temperature and pressure, and ERH (%) is equilibrium relative humidity at which a solution or solid substrate neither gains nor loses moisture to the atmosphere.

Water activity can, therefore, be seen to be a measure of the ability of water to evaporate from a substrate and humidify the immediate environment, and is measured in the range 0–1.0 with 1.0 representing the a_w of pure water. Most importantly it should be noted that a_w is a function of temperature, and for a given substrate and moisture content, a_w will increase with increasing temperature. This is primarily the consequence of the general increase in thermal motion (Multon 1988).

2.1.3 Water Potential

Water availability may also be expressed as water potential (ψ). Water potential approaches the concept of water

availability in terms of the thermodynamics of systems, and considers the movement of water along gradients of potential energy. The term “water potential” is actually an abbreviation of “potential energy of water” and is defined as “the free energy of water in a system relative to the free energy of a reference pool of pure free water having a specified mass or volume,” and is measured in J/m³ or Pa (Papendick and Mulla 1985). The reference state of pure free water is assigned zero. Water which is “constrained” in a system, i.e., the constitutive, adsorbed and (to a lesser extent) absorbed water is, therefore, at a lower (negative) water potential, and any microorganism must expend energy to lower internal water potential values relative to the exterior to make water available. The numerical value of water potential may be related to water activity using the formula:

$$\psi = (RT/V_w) \ln a_w \quad (\text{Papendick and Mulla 1985}).$$

where R is gas constant, T , absolute temperature (K), and V_w is molecular weight of water.

Although ψ and a_w are interconvertible by this expression, measurement of the separate components that make up total water potential cannot be carried out using water activity measurement, since water activity is not a sufficiently sensitive parameter.

2.1.4 Moisture Sorption Isotherms

The relationship between water content and a_w in a given substrate may be usefully expressed by moisture sorption isotherms. These are curves produced by plotting water content against water activity, or ERH at constant temperature (hence, the term “isotherm”). They are highly specific to substrate type and condition but generally sigmoidal in shape show a steep rise in the curve above 80% ERH. The importance of moisture sorption isotherms is that they establish the relationship between moisture content and water availability for specific product types and, therefore, allow water availability to be set to safe levels from knowledge of moisture content alone. For most stored seed products safe moisture levels will typically correspond to water activity levels of around 0.7 water activity or 70% ERH. The actual moisture content which it corresponds to will vary greatly between seed types, and particularly between starchy and oily types. Moisture sorption isotherms are complicated by a hysteresis effect that influences the position of the curve depending on whether the material is being wetted or dried as each equilibrium point is plotted. This effect may account for a difference of as much as 0.5% (Pixton 1967) and always shows a higher water activity for a given moisture content where water is being sequentially added. This indicates that the “history” of the material is important and probably reflects irreversible and permanent changes occurring after wetting.

2.2 Temperature

Fungi differ widely in the range of temperature over which growth can occur and those conditions optimum for growth (Magan 1997). For example, *Penicillium* species such *P. aurantiogriseum* and *P. verrucosum* can grow over the range -4 to $+35^{\circ}\text{C}$ while *Aspergillus fumigatus* has a very wide tolerance of 10 – 55°C and *Humicola lanuginosa* in the range 30 – 60°C . The majority of fungi involved in deterioration in stored grain ecosystems thrive over the range 10 – 40°C with optima in the range 25 – 35°C . Lowering temperature reduces the metabolic activity of fungi and the grain, enabling longer mold-free storage periods. However, moist grain can be prone to slow deterioration by the genera *Alternaria*, *Penicillium*, and *Fusarium*, with species in each able to produce mycotoxins. Temperature of grain is a good indicator of quality during storage. Pockets of moisture can allow initiation of fungal activity that produces metabolic heat resulting in a succession of fungi becoming dominant and ending with spontaneous heating and dominance by thermotolerant/thermophilic fungi and actinomycetes (Lacey and Magan 1991).

2.3 Gas Composition

While fungi involved in biodeterioration of grain are considered to be obligate aerobes, many are actually microaerophilic, being able to survive and grow in niches where other species cannot grow and, thus dominate specialized grain ecosystems. In many cases, decreasing O_2 to $<0.14\%$ is required before growth can be substantially reduced. Increasing CO_2 to $>50\%$ is required for inhibition of growth (Magan and Lacey 1984a). Some species, e.g., *P. roqueforti*, are able to grow and infect grain at $>80\%$ CO_2 provided at least 4% O_2 is available. The use of integrated postharvest systems for prevention of deterioration entails modifying O_2 and CO_2 simultaneously and the use of (O_2 free) N_2 . The tolerance to low O_2 and high CO_2 is also influenced by interactions with grain water availability. The treatment is more effective if the grain is dry. Controlled atmosphere storage is used for both control of molds and insects in moist stored seed systems. Regimes sufficient for molds may not, however, be effective against some storage insects that can survive and grow over a wide ERH range.

In summary, from the whole range of factors that can affect the colonization of seeds by microorganisms, a_w is the most important single factor limiting their activity. Each microorganism has a specific range over which they can develop. Fungi are the most important group of microorganisms with capacity to colonize seeds because specialized groups can grow at intermediate and low a_w levels. Fungi contribute greatly to seed losses, either alone or together with insects. Because the environment and other factors can change during seed/grain filling, harvest, and storage the

fungal community can be very different colonization of grain pre- and postharvest are described separately.

3 POSTHARVEST FUNGAL COLONIZATION OF CEREAL GRAINS

3.1 Factors

Conditions in the stored grain are more stable and controlled than those in the field. Stored cereal grain offers a good ecological niche for fungi. The a_w , temperature, O_2 level, interactions with other organisms like insects, chemical preservatives, and storage time are considered the most important factors that control the growth of fungi in grains but cannot be considered in isolation. All these factors interact with each other during storage. For instance, fluctuations in temperature can cause condensation of water that increases the water activity in pockets of stored grain. Damage to the kernels not only increases susceptibility to fungal invasion, especially if this occurs near the embryo, but also increases the likelihood of visible molding (Perez et al. 1982; Tuite et al. 1985; Lacey et al. 1991).

3.2 Mycoflora

The number of fungal species that can be found colonizing stored grain is very wide. These represent a high number of genera and include psychrotolerant, mesophilic, thermophilic, xerophilic, and hydrophilic species (Hill and Lacey 1983; Lacey et al. 1980; Magan and Lacey 1984a,b,c). The most characteristic of these are species belonging to the genus *Aspergillus* and *Penicillium*. In addition, it is possible to find species of *Eurotium*, *Fusarium*, *Rhizopus*, and thermophiles like *Talaromyces thermophilus*, *Rhizomucor pusillus*, and *Thermomyces lanuginosus* (Figure 2). All these species are widespread in their occurrence throughout the world and they cover almost the whole range of environmental conditions likely to be found in stored grain.

The a_w and temperature are very important factors that determine the mycoflora present in stored grains. The fungi present in the field such as *Alternaria* species that need high levels of a_w to grow, decrease in importance when the grain is stored and subjected to processing practices directed to decrease the water activity. On the other hand, genera such as *Aspergillus*, *Penicillium*, and *Eurotium* that have a low importance in the field, become very important during the storage of the grains. Another factor that determines the mycoflora in grain is the oxygen concentration, because the fungi are generally very sensitive to low oxygen concentrations. Under these conditions Magan and Lacey (1984) reported that *Fusarium culmorum*, *P. roqueforti*, and *A. candidus* were the most tolerant, which is consistent with the fungal community found on cereals under airtight storage.

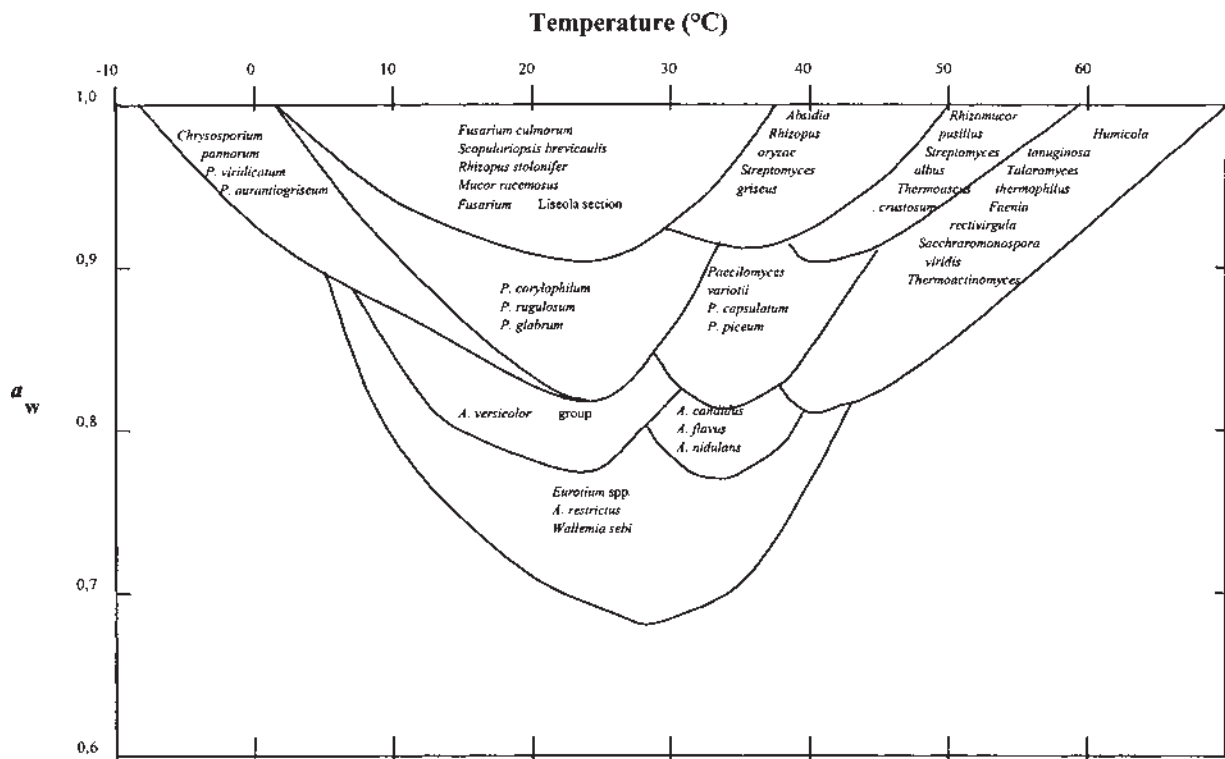


Figure 2 Succession of dominant fungi found in stored grain depending on its initial water activity and temperature [adopted from Lacey et al. (1980)].

Both climatic conditions and geographical location can explain the differences found in the mycoflora of different grains in the field. During storage, however, the mycoflora is very similar for all grains (Pelhate 1988). Many of the species found on maize, sorghum, rice, and other cereals in tropical climates are the same as those found in temperate regions except for a small number of additional species and, perhaps, a greater frequency of *Aspergillus* and fewer or different *Penicillium* spp.

4 ECOLOGY OF GERMINATION, GROWTH, AND MYCOTOXIN PRODUCTION

To colonize grain, fungi need to be capable of germinating and growing under the prevailing environmental conditions found in the substrate. If conditions are appropriate, establishment can also result in the accumulation of mycotoxins. Consequently, it is very important to understand the interacting ecological conditions that can avoid fungal colonization of the grain. The main environmental factors that affect germination, growth, and mycotoxin production by fungi are a_w , temperature, and intergranular atmosphere. Species differ in their ecological requirements and in their tolerance of low a_w and marginal temperatures. Germination of conidia and mycelial growth of isolates of some

Penicillium, *Aspergillus*, and *Fusarium* spp. isolated from maize grain have been found to be significantly influenced by a_w , temperature, and their interactions. The effects of a_w and temperature on germination can be quantified in two ways: (a) the minimum a_w for germination and (b) the lag time before germination.

Fungi normally found in stored grain can germinate at lower a_w levels than the fungi present in the field, especially in temperate grains (Table 1). Changes in tolerance to low a_w occur when changing temperature level. *A. niger* germinates only down to 0.95 at 10°C, but down to 0.90 at 15°C, and to 0.80 at 25°C. *P. hordei* germinates down to 0.90 at 5°C, but down to 0.85 at 25°C. *F. verticillioides* germinates only down to 0.96 at 5°C, but down to 0.88 at 25°C (Marin et al. 1996, 1998a).

In general, the lag time before germination increases with decreasing a_w . With water readily available (>0.98) the lag time can range from a few hours to a few days, while at very low a_w it can extend for months or even years (Magan and Lacey 1984a; Marin et al. 1998a; Pitt 1975). For example, the shortest lag phases for species of *Aspergillus*, *Penicillium*, and *Fusarium* are at 0.994 and 0.95 a_w over a wide range of temperatures (Marin et al. 1996, 1998a) (Figure 3). At marginal temperatures the lag phases increase, more markedly for *P. aurantiogriseum* and *P. hordei* at $>30^\circ\text{C}$ and $<15^\circ\text{C}$ (Marin et al. 1998a). The capacity to germinate

Table 1 The minimum water activity for germination and growth of some field and storage fungi on wheat extract agar at 25°C

Fungal species	Germination	Growth
<i>A. alternata</i>	0.85	0.88
<i>C. cladosporioides</i>	0.86	0.88
<i>C. herbarum</i>	0.85	0.90
<i>E. purpurascens</i>	0.88	0.99
<i>F. culmorum</i>	0.87	0.90
<i>P. brevicompactum</i>	0.80	0.82
<i>P. aurantiogriseum</i>	0.80	0.83
<i>P. hordei</i>	0.80	0.83
<i>P. roquefortii</i>	0.83	0.83
<i>E. amstelodami</i>	0.72	0.73
<i>E. repens</i>	0.72	0.75
<i>A. fumigatus</i>	0.94	0.94
<i>A. versicolor</i>	0.76	0.78

Source: Magan and Lacey (1984a).

very fast can be an advantage in competing effectively with other fungi for nutrients in the grain and exclude other competitors. Also, conidia can survive for long periods of time depending on the relative humidity and temperature. All of this information gives us an idea about the potential capability to colonize the stored grain ecosystem if the conditions change and become favorable for fungal development.

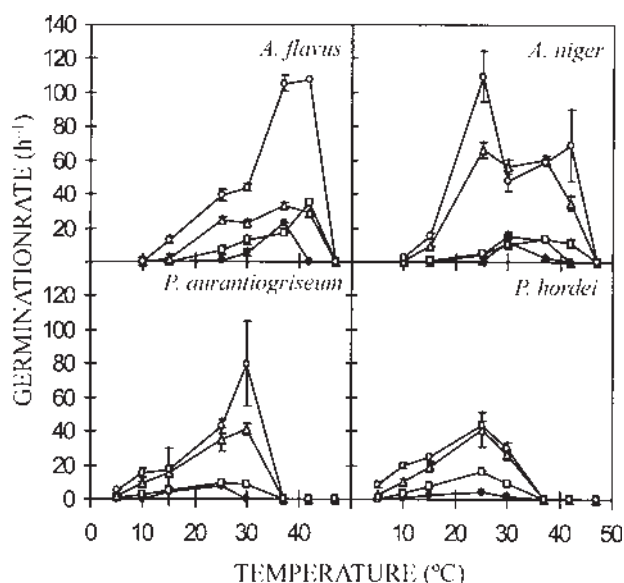


Figure 3 Effect of water activity and temperature on the germination rate of *A. flavus*, *A. niger*, *P. aurantiogriseum*, and *P. hordei* on MMEA. Water activity levels are 0.994 (○), 0.95 (△), 0.90 (□), 0.85 (●), and 0.80 (▲). Error bars show standard error of estimated parameters [from Marin et al. (1996)].

Successful spore germination results in the formation of extending hyphae that are able to colonize the substrate. Hydrolytic enzymes are produced to utilize the rich nutritional substrate. Water activity and temperature are very important factors impacting on the level of infection of the grain and the extent of colonization. The interaction between these factors is critical in developing an understanding of the ecology of individual species, their interrelationship with one another, and their role in initiation of molding.

The range of a_w /temperature conditions for germination is generally found to be wider than that for mycelial growth, as recently shown for fumonisin-producing species of *Fusarium* from maize, and for other *Aspergillus* and *Penicillium* spp. (Magan and Lacey 1984a; Marin et al. 1996). Changing a_w at different steady-state temperatures affected growth rates of all the species examined (Marin et al. 1996, 1998a). *Fusarium* species grow faster on irradiated maize at 25°C than at 15°C. Maximum growth rates of about 3.6, 3.5, and 10.4 mm/d were obtained for *F. verticillioides*, *F. proliferatum*, and *F. graminearum*, respectively, at 0.98 a_w and 25°C. However, these three species had a growth rate of about 0.4, 0.5, and 1.2 mm/d at 0.93 a_w and 15°C, respectively (Marin et al. 1998b).

While most fungi grow optimally close to 1.00 a_w at their optimum growth temperature, others grow better at lower a_w . For instance, *Eurotium amstelodami*, *E. repens*, *A. niger* and *A. versicolor* and *A. ochraceus* all grow faster at 0.90–0.95 a_w at optimum temperatures (Ayerst 1965; Magan and Lacey 1984a; Ramos et al. 1999a,b). Some *Penicillium* spp. also grow better nearer to 0.98 than at 1.00 a_w (Hocking and Pitt 1979). However, at marginal temperatures for growth, optima are almost always close to 1.00 a_w . The effects of a_w and temperature on fungal growth and other activities in stored grain may be further modified by changes in the concentration of O₂ and CO₂ in the intergranular atmosphere resulting from respiration (Magan and Lacey 1984b), and the addition of preservatives (Marin et al. 1999b).

The production of mycotoxins has been studied mostly at optimum a_w and temperature levels on either rich laboratory media or on autoclaved wheat, barley, or rice. Few studies have considered the effects of environmental factors, especially water stress on mycotoxin production. The conditions of a_w /temperature that permit mycotoxin production differ from those for the fungal growth (Table 2). For instance, *F. verticillioides* and *F. proliferatum*, at minimum a_w /temperature for growth (e.g., 0.89–0.91 a_w) did not produce detectable levels of FB₁ (Marin et al. 1999a). The minimum a_w allowing growth of 12 species of fungi was usually lower and the temperature range wider than those permitting aflatoxin, patulin, penicillic acid, ochratoxin, or fumonisins production (Marin et al. 1999a,c; Northolt 1979). Moreover, the effect of these factors can differ for two toxins produced by the same species. For instance, ochratoxin production by *A. ochraceus* on poultry feed was greatest at 30°C and 0.95 a_w while penicillic acid production was favored by 22°C and 0.90 a_w .

Table 2 Minimum water activity for growth and toxin production by some toxigenic fungi

Fungal species	Mycotoxin	Minimum a_w	
		Growth	Toxin production
<i>A. flavus</i>	Aflatoxin	0.78–0.84	0.84
<i>A. parasiticus</i>		0.84	0.87
<i>A. ochraceus</i>		0.77	0.85
<i>P. aurantiogriseum</i>	Ochratoxin	0.82–0.85	0.87–0.90
<i>P. viridicatum</i>		0.80–0.81	0.83–0.86
<i>A. ochraceus</i>		0.77	0.88
<i>P. aurantiogriseum</i>	Penicillic acid	0.82–0.85	0.97
<i>P. patulum</i>		0.81	0.95
<i>P. expansum</i>		0.82–0.84	0.99
<i>A. clavatus</i>	Patulin	—	0.99
<i>F. verticillioides</i>		0.88	0.93
<i>F. proliferatum</i>		0.88	0.93

4.1 Interaction Between Grain Fungi, Environmental Factors, and Niche Occupation

Fungi seldom occur on grains in isolation, but usually as a mixed consortium of bacteria, yeasts, and filamentous fungi. It is thus inevitable that interspecific and intraspecific interactions will occur depending on the nutritional substrate of the grain. Furthermore, environmental factors may exert a selective pressure influencing community structure and dominance of individual species. It is important to understand the type of interactions that occur between fungi under different environmental regimes in grain to enable better prediction of not just dominance by key spoilage fungi, but also the potential for production of mycotoxins. Wicklow (1988) used the *in vitro* interactions between hyphae of different fungi based on: (a) intermingling of hyphae, (b) inhibition on contact, (c) inhibition at a distance, and (d) dominance by one species over another on contact, and (e) inhibition by one species at a distance with the dominant species continuing to grow. They used these categories to develop an index of antagonism by giving numerical scores to each interaction type. This enabled antagonistic interactions between *A. flavus* and a range of species to be identified. However, these studies did not examine the dynamics of interacting environmental factors and dominance of species.

Subsequent studies by Magan and Lacey (1984c, 1985) modified this scoring system to give a higher numerical score to fungi able to dominate *in vitro* than antagonism and developed an index of dominance (I_D) to assist with interpreting patterns of colonization and dominance in grain ecosystems. The I_D was found to significantly change with a_w and temperature and also with nutritional grain substrate. Of the 15 species, the most competitive species in wheat grain in United Kingdom were found to be *P. brevicompactum*, *P. hordei*, *P. roqueforti*, *A. fumigatus*, and *A. nidulans*.

Decreasing the a_w conditions increased competitiveness of *P. brevicompactum*. Only *F. culmorum* could compete with storage molds, at >0.93 – 0.95 a_w . They also found that rate of growth was not directly related to dominance. Previously, Ayerst (1965) had suggested that speed of germination and growth were key determinants of colonization of nutrient rich matrices such as grain. The I_D approach has been adapted over the years for many food-based ecosystems.

More recently, alternative approaches have been utilized to try and understand the relative competitiveness of different species within fungal communities colonizing grain. It was suggested by Wilson and Lindow (1994a,b) that the coexistence of microorganisms particularly on plant surfaces may be mediated by nutritional resource partitioning. Thus *in vitro* carbon utilization patterns could be used to determine niche overlap indices (NOI) and, thus the level of ecological similarity. Based on the range of similar c-sources utilized and those unique to an individual isolate of species they suggested that NOI values of >0.9 were indicative of coexistence between species in an ecological niche, while scores of <0.9 represented occupation of separate niches. This approach was modified by Lee and Magan (1999a,b) and Marin et al. (1998c) to include a multifactorial approach by including water availability and temperature into the system. This approach demonstrated that based on utilization of maize c-sources the NOIs for *F. verticillioides* and *F. proliferatum* were >0.90 at >0.96 a_w at 25 and 30°C, indicative of coexistence with other species such as *Penicillium* species, *A. flavus* and *A. ochraceus*. However, for some species pairing with *F. verticillioides* resulted in NOI values <0.80 indicating occupation of different niches. Interestingly, no correlation could be found between I_D and NOI methods. The results suggested that niche overlap was in a state of flux and significantly influenced by both temperature and water availability. The nutrient status is very important as Lee and Magan (1999) demonstrate that comparison of c-sources in a standard BIOLOG test plate with only those relevant to maize

grain gave very different results in terms of niche size and NOI under different environmental conditions. This approach confirms that interactions and dominance are dynamic and not static and emphasizes the importance of taking account of such fluxes in any integrated approach to control the activity of spoilage molds in the stored seed ecosystem.

5 RELATIONSHIP BETWEEN MOLD DETERIORATION OF CEREAL GRAIN AND CALORIFIC LOSSES

It is surprising to note that few studies have been conducted if poor postharvest practices can result in the nutritional quality loss of grain due to the activity of spoilage fungi (Sinha 1982). Different grains have different levels of intrinsic calorific values and, thus deterioration by different spoilage fungi will affect these values to different extents. Elegant studies by Demenyk and Sinha (1988), Sinha (1995), and Sinha et al. (1986) demonstrated that the bioenergetics of insects feeding on different grain types enables energy budgets to be established and provided useful information on the impact that pests have on actual calorific value of stored cereals. However, in stored grain ecosystems factors such as a_w , temperature, gas composition, and level of fungal contamination will all have a significant impact on calorific losses and provide a useful link with quantifiable levels of dry matter losses for different cereals.

Studies by Prasada and Prasad (1982) reported changes in the calorific value of linseed (*Linum usitatissimum*; $L = 1.896 \text{ kJ/g}$) due to seed-borne infection by spoilage fungi. They found that *A. niger* infection resulted in maximal calorific losses of 25.2% within 15 days, and almost 50% in 30 days at 28°C on surface sterilized seeds. Losses from autoclaved and inoculated seeds were higher. However, water availability was not monitored or controlled. More recent studies where both a_w and temperature were carefully controlled have examined the effects caused by the mycotoxin producing species of *F. verticillioides* and *F. proliferatum* and *A. ochraceus* (Marin et al. 1999c; Ramos et al. 1999a,b). The *Fusarium* spp. were found to maximally cause calorific losses of maize based substrates ($= 19.69 \text{ kJ/g}$) at 0.98 a_w (17–64%) after 4 weeks depending on temperature of incubation, but negligible at 0.92 a_w (0–9%). An inverse correlation was found between calorific losses and fungal biomass and fumonisin production. For *A. ochraceus* calorific losses were maximal at 0.95 a_w and 20–30°C on similar maize-based substrates. Losses were in the region of 10–17% over 4 weeks with much lower losses at 0.85 a_w (0–7%). There was a direct relationship between increase in fungal biomass and calorific losses. As each grain type has a different intrinsic calorific value it is critical that these studies are expanded to include rice and other staple cereals to enable the relationship between the activity of spoilage fungi in stored cereals to be quantified both on the basis of the significance of nutritional losses and in terms of dry matter losses.

6 RESPIRATION AND DRY MATTER LOSSES

Harvested grain carries a wide range of bacterial and fungal contaminants. Depending on effectiveness of storage conditions, and the climatic region of the world the level and type of contamination will vary. Grain itself and the microbial contaminants respire slowly when stored dry. However, if the water availability is increased to 15–19% moisture content ($= 0.75\text{--}0.85 a_w$) predominantly spoilage fungi, particularly *Eurotium* spp., *Aspergillus*, and *Penicillium* species grow resulting in a significant increase in respiratory activity, resulting in increased temperature and sometimes spontaneous heating that results in colonization by thermophilic fungi and actinomycetes (Fleurat-Lessard 2002; Lacey and Magan 1991). The chemical process involved in heat generation is predominantly aerobic oxidation of carbohydrates such as starch. The energy is released by the following equation:



Heating occurs when this energy is released faster than it can escape from the cereal substrate. In contrast, little energy is released in anaerobic respiration and little or no heating occurs in the absence of oxygen. The requirement for oxygen increases with temperature to a maximum of 40°C but does not decrease greatly until the temperature exceeds 65°C. At this temperature, microbial growth is largely inhibited and heating results from exothermic chemical oxidation. Thus, the respiratory quotient may be 0.7 to 0.9 up to 65°C but less than 0.5 at higher temperatures.

By utilizing the respiratory quotient CO_2 production can be translated into dry matter loss. Typically, complete respiration of carbohydrates gives a respiratory quotient, i.e., ratio of O_2 consumed to CO_2 produced of 1.0, and it has been calculated that 14.7 g CO_2/kg grain will be released for every 1% loss of grain dry matter. During anaerobic fermentation, only about 0.493 g CO_2 is evolved from a kilogram grain for every 1% dry matter loss. Alternatively, a respiratory quotient below 1.0 may result from lipid or protein metabolism. For example, tripalmitin has a quotient of 0.7. The higher the CO_2 production, the shorter the safe storage period without loss of dry matter. Studies by Jonsson et al. (2000) utilized respiratory rates over a wide range of a_w levels and temperatures to examine development of molds such as *P. verrucosum* in stored grain and also effects on germinability, fungal biomass, and maximum safe storage times in days. They suggested that the maximum storage time without mold growth was probably halved if moisture content at harvest was increased by 1–3% ($= 0.05 a_w$) or if storage temperature was increased by 5°C. Fleurat-Lessard (2002) in his excellent review has suggested that for the modeling and prediction of global quality changes the rate of CO_2 production can be used as a “storability risk factor.”

The ratio of contribution of spoilage molds and grain to total respiration has been argued for many years. A range of

studies has demonstrated that grain deterioration and by implication dry matter loss is predominantly determined by fungal activity. Wheat deterioration has been measured and models developed based on germination rates, visible mold growth, or respiration of grain and microorganisms (Fleurat-Lessard 2002). Kreyger (1972) pointed out, from previous work with wheat that dry matter loss was unimportant, provided there was no visible molding. However, his own work showed that barley of 24% water content ($=0.94 a_w$) stored at 16°C for 10 weeks lost 2% dry matter with visible molding. With maize showed that fungal invasion and aflatoxin content could be unacceptable before the grain had lost 0.5% dry matter and mold growth became visible (Seitz et al. 1982). Latif and Lissik (1986) proposed a model for respiration based on the rate of dry matter breakdown, but was not related to important environmental factors such as a_w and temperature. This worked suggested that Kreyger's assumptions were not completely accurate. White et al. (1982) noted that 0.1% was unacceptable for first grade wheat and proposed an absolute level of 0.04%. However, when 55 days safe storage was predicted for grain stored at 18.4% mc ($=0.86 a_w$) visible molding occurred after 23 days of storage. Recently, Karunakaran et al. (2001) determined deterioration rates in wheat stored at constant or step decreases in temperature. Deterioration rates at 17% mc for wheat were 5, 7, and 15 days at 35, 30, and 25°C, respectively. Interestingly, they found that respiration rates of 17–19% mc wheat at 25°C increased while germination percentages decreased with storage time. Dry matter losses were about 0.05% and visible mold was observed when dry matter loss was about 0.1% at these w.c.s.

There are problems with the use of visible molding as a criterion of deterioration (Hamer et al. 1991; Lacey et al. 1994). While Kreyger (1972) used this extensively, a clear definition was not produced. Many workers have questioned this subjective index of the safe storability of grain (Hamer et al. 1991; Lacey and Magan 1991). Magan (1993) pointed out as an early indicator, microscopic growth may be a more effective measurement of fungal activity than visible molding.

7 BIOCHEMICAL TESTS

A wide variety of methods have been used to quantify the fungal activity in stored grain. Chitin, ergosterol, adenine triphosphate, immunofluorescence, immunoassays, and DNA probes have all been developed (Fleurat-Lessard 2002; Magan 1993). Since, ergosterol is the predominant sterol in most spoilage fungi (ascomycetes and deuteromycetes) and not found in insect pests it has been utilized extensively as an indicator of whether deterioration has occurred in grain. The method was first described by Seitz et al. (1977) and can now be performed relatively quickly and routinely using simple extraction and HPLC. It has thus been used extensively for the *in vitro* quantification of biomass of spoilage fungi which demonstrated that this does change with culture age (Marfleet

et al. 1991). Cahagnier and Richard Mollard (1991) suggested that the ergosterol content in storage fungi was not significantly affected by environmental factors such as a_w . They thus suggested that ergosterol could be used as an “index” of the level of fungal biomass and the length of storage of the grain. Tothill et al. (1993) showed that there was a significant positive correlation between ergosterol content and total CFUs at 0.95 a_w , while in grain of 0.85 a_w there was no significant correlation. Grain inoculated with individual species (*Alternaria alternata*, *E. amstelodami*, and *P. aurantiogriseum*) at 0.95/0.85 a_w and 25°C showed a significant correlation between CFUs and ergosterol although the content for an individual species varied considerably. Overall, levels of $<5\text{--}6\text{ }\mu\text{g/g}$ can be found in fresh wheat grain, with that having microscopic growth containing about $7.5\text{--}12\text{ }\mu\text{g/g}$. This correlates with a threshold of 10^5 CFUs/g grain as a spoilage threshold indicator. Fleurat-Lessard (2002) has suggested that perhaps modeling of ergosterol production rates under different environmental conditions using sigmoid curves similar to those used for insect population dynamics may enable the use of an ergosterol index in the future when correlation models become available. It may also be possible to use both ergosterol and the production of mycotoxins in predicting potential environmental factors over which spoilage/mycotoxins may be produced. Two-dimensional models for growth and fumonisin production have already been developed (Marin et al. 1999a,b,c) and such information may be useful in further development of predictive models for risk assessment of spoilage and toxin contamination. Perhaps, modeling of cumulative ergosterol production by spoilage fungi and associated mycotoxins in relation to a_w , temperature, time, gas composition (modified atmosphere storage), and time may allow more effective and precise risk assessment of mold contamination and mycotoxin occurrence to the consumer.

8 ENZYME CHANGES AS AN INDICATOR OF DETERIORATION

Changes in grain enzyme concentrations, e.g., amylases, due to fungal deterioration are important as they have an impact on processing and bread making quality of flour and dough. However, studies which examined α -amylase, β -amylase, and total amylases of wheat found no correlation with the time to microscopic/visible molding (Magan 1993). Fleurat-Lessard (2002) suggested that for both wheat and malting barley enzyme changes are too small and occur too late as functions of storage conditions and duration, especially with regard to incorporation in a model for decision support systems.

However, there are other studies which suggests the opposite. Fungi colonizing the rich grain substrate under conducive environmental conditions produce a battery of hydrolytic enzymes for degrading the grains and causing the dry matter losses discussed earlier in this Chapter. Flannigan

and Bana (1980) and Magan (1993) showed that a_w and temperature affect the production of enzymes by fungi during grain colonization, including cellulases, polygalacturonase, pectin methyl esterase, 1-4- β -glucanase, β -glucosidase, β -xylosidase, and lipases. Jain et al. (1991) were the first to demonstrate that by using chromogenic 4-nitrophenol substrates in an ELISA well format, rapid quantification could be carried out for a range of hydrolytic enzymes, provided that substrates were available for them. They demonstrated that in both barley and wheat grain at different a_w levels (0.85, 0.90, and 0.95) significant increases in *N*-acetyl- β -D-glucosaminidase were produced when compared to nonmolded dry harvested grain. Grain inoculated with the xerophile *E. amstelodami* also showed marked increases in α -D-galactosidase.

Magan (1993) extended this and examined stored dry grain with that at different a_w levels and temperatures of incubation. This showed that a significant change in the production of some enzymes was evident at times of microscopic and visible molding. Of seven enzymes examined significant changes in β -D-glucoaminidase, β -D-galactosidase, and β -D-glucosidase were observed by the time microscopic growth had occurred. Similar results were obtained with fumonisin producing *Fusaria* (*F. verticillioides*, *F. proliferatum*) by Marin et al. (1998d). Changes could be monitored within 72 h of storage. They also suggested that these enzymes could be used as an early indicator of infection of maize grain by such species and that these enzymes were important in enabling rapid colonization over a wide range of environmental factors. Recent work by Keshri and Magan (1998) and Keshri et al. (2002) have also suggested similar hydrolytic enzymes are an early indicator of fungal activity *in vitro* on wheat flour-based media and in bread substrates. Thus, potential does now exist for the use of such relatively simple enzyme assays to be used as a possible tool for early detection of fungal activity in cereal grain.

9 VIABILITY AND DEGRADATION OF GRAIN

Fungal activity inevitably leads to deterioration and loss in viability of grain to be used as seed. Whether seed death occurs directly due to fungal activity or due to degradation of key grain nutritional components is, however, unclear. Under some circumstances fungi have been shown to be key components in deterioration of viability. The grain embryo is sometimes preferentially invaded by some fungi, especially species of *Aspergillus* and *Penicillium*. In most early classical work it was demonstrated that spoilage of stored grain resulted in seed viability being rapidly reduced to practically zero. Hill and Lacey (1983) found a linear relationship between initial moisture content and percentage viability. However, few studies in the last decade have examined the detailed changes in nutritional components of grain. Thus, we are reliant on other earlier work for such information.

9.1 Carbohydrate Degradation

Starchy cereal grains contain 75% carbohydrates that are hydrolyzed during respiration by amylases. As indicated earlier, while fungal invasion does lead to increases in reducing sugars these changes are not early enough as an early indicator of deterioration (Magan 1993).

9.2 Protein Degradation

Total proteins represents about 14.5% of the grain dry matter in wheat and quality can vary considerably (Aspinall and Greenwood 1965). Protein type, quality, and amount are all important for bread and baking quality. It is thus surprising that, although protein quality is important to human nutrition few studies have attempted to analyze in detail the impact that fungal activity might have on this component. No changes in total protein during spoilage and heating, while others found an increase in total protein. Colonization of wheat grain by fungi such as *A. tenuis*, *A. flavus*, *A. niger*, and *F. solani* resulted in an increase in total protein although a_w or temperature considerations were not considered. More research is needed on the potential changes, particularly in storage proteins that may occur due to fungal invasion of grain.

9.3 Lipid Degradation

Starchy grains contain about 2.5% lipids while oil rich seeds contain much more (Aspinall and Greenwood 1965). Lipids can be degraded endogenously and via fungal invasion, both by oxidation and by hydrolysis, with lipooxygenases and lipases, respectively (Zeleny and Coleman 1938). Usually an increase in free fatty acids in the seeds is indicative of utilization by spoilage fungi. Indeed Magan et al. (1993) demonstrated that free fatty acid values for different spoilage fungi varied considerably with a_w and temperature in *in vitro* studies with rape seed oil and with rape seed. Farag et al. (1985a,b) had earlier showed that fungal lipases degraded different classes of lipids by degrading triglycerides to mono/diglycerides and free fatty acids.

10 FUNGAL VOLATILES AS AN EARLY INDICATOR OF DETERIORATION

The production of volatiles by fungi when colonizing grain has been quantified and the key components identified *in vitro* and *in situ* (Kaminski et al 1974; Magan and Evans 2000; Schnurer et al. 1999). Key volatiles indicative of spoilage include 3-octanone, methylheptanone, 2-methyl-1-propanol, cyclohexanone, trimethylbenzene, undecane, naphthalene, and dodecane. Previous *in situ* studies have demonstrated that measurement of such volatiles show promise in early

detection of deterioration of grain in stores (Tuma et al. 1989). The rapid development of electronic nose technology, which uses a variety of sensor arrays for giving qualitative information on changes in patterns of volatile production, has enabled this technology to be examined in detail for practical applications in the grain commodity chain. Evans et al. (2000) demonstrated that it was possible to classify grain samples in real time into categories of good quality, molded, or suspect grain using an automated computer controlled system coupled with radial based models. The potential for using odor classification from grain as a spoilage indicator has also been demonstrated (Magan and Evans 2000). Work has also shown that e.nose technology could be used for differentiating between different isolates of the same spoilage species on the basis of qualitative volatile production patterns, which may differ due to specific biosynthetic pathways for isolates producing mycotoxins in *Fusarium* section Liseola species (Keshri and Magan 2001); and in naturally contaminated grain samples (Olssen et al. 2002).

11 BIOLOGICAL CONTROL OF SPOILAGE IN MOIST CEREALS

Storage of moist cereals for animal feed is quite common in temperate climatic regions. Usually to achieve this, cereals are kept sealed to allow a build up of CO₂ to inhibitory concentrations with very low O₂ concentrations. Sometimes, improper sealing enables spoilage to proceed rapidly, especially by microaerophilic species such as *P. roqueforti*, *P. carneum*, and *P. paneum*. This results in poor quality animal feed with potential contamination with mycotoxins (Petersson and Schnurer 1998). Recent studies in Sweden have approached this problem by the use of antagonistic and competitive yeasts such as *Pichia anomala* which can be inoculated onto wheat, barley, or oats prior to storage. This yeast effectively colonizes the stored cereal and prevents spoilage molds from causing deterioration in feed grain quality. *P. anomala* has been found to be effective at controlling growth of *P. roqueforti* in moist cereals (Petersson and Schnurer 1998). Recent studies indicate that antagonistic yeasts such as *P. anomala* may also affect ochratoxin A accumulation by *P. verrucosum*, and other important toxigenic species in cool temperate climates (Petersson et al. 1998). This approach has not been examined for moist tropical cereals destined for animal feed where often spoilage fungi can invade preharvest and thus make postharvest control more difficult. This could, however, be a promising area for future commercialization of natural inoculant treatments, especially for animal feedstuffs free of mycotoxin contamination.

12 CONCLUSIONS

In many areas of research, a better understanding of the science of the spoilage fungi and their role in deterioration of

seeds has been achieved in the last decade. Certainly, the key role of spoilage molds, the conditions under which they may produce mycotoxins, early detection systems and predictions of dry matter losses, and an understanding of the complex interactions that occur in the stored seed ecosystem are better understood. In certain areas, particularly with regard to quantifying nutritional losses generally and quantifying degradation of grain components needs to be moved forward in the coming years. It is critical that such studies are linked to and correlated with other measures of quality loss of stored seeds, especially staple grains. This would enable us to realize the goal of developing realistic and accurate decision support systems for effective conservation of seeds postharvest. In the coming years we will need to build on this sound foundation in developing strategies for the prevention of spoiled grain and mycotoxins entering the human and animal food chain.

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Mycotoxins

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1 INTRODUCTION

1.1 Mycotoxins, Mycotoxicosis, and Mycotoxicology

Mycotoxin is a convenient generic term describing the toxic secondary metabolites produced by fungi. “Myco” means fungal (mold) and “toxin” represents poison. They encompass a considerable variety of low molecular weight compounds with diverse chemical structures and biological activities. Some mycotoxins could also be toxic to plants or other microorganisms; but these compounds are not classified as antibiotics of fungal origin. Like most microbial secondary metabolites, the benefit of mycotoxins for the fungi themselves is still not clearly defined. In considering the effects of mycotoxins on animals, it is important to distinguish between “mycotoxicosis” and “mycosis.” Mycotoxicosis is used to describe the action of mycotoxin(s) and is frequently mediated through a number of organs, notably the liver, kidney, lungs, and the nervous, endocrine, and immune systems. On the other hand, “mycosis” refers to a generalized invasion of living tissue(s) by growing fungi (CAST 2003; Chu 1998). Due to their diverse chemical structures, mycotoxins may exhibit a number of biological effects, including both acute and chronic toxic effects as well as carcinogenic, mutagenic /genotoxic, teratogenic, and immunotoxic effects (Bhatnagar et al. 2002; Chu 1998; 2002; Wogan 1992). Modulation of the animal immunosystem, either immunosuppressive (most often) or immunostimulatory, also plays an important role for the overall toxic effects (Bondy and Pestka 2000). The interaction of mycotoxins with cellular macromolecules plays a dominant

role in their toxic actions (Chu 2002; Hussein and Brasel 2001). Recent studies on the effect of mycotoxins on apoptosis have further revealed their mode of action at the cellular level (Chu 2002). The complexity of the biological effects of mycotoxins has led to a scientific discipline named “mycotoxicology” for the study of various issues that are encountered with this group of toxins.

Mycotoxins have been the subject of many reviews, and recently several comprehensive reviews have discussed various aspects of this topic (Bhatnagar et al. 2002; CAST 2003; Chu 2002; Scudmore 2000). Due to a limitation of space, in this chapter we have tried to cover, as much as possible, the most recent advances achieved in the field, and referred primarily to reviews listing all the previous landmark references in the field.

1.2 Historical Background

Although mycotoxicoses have been known for a long time, identification of a specific mycotoxin as a causative agent for these illnesses was unknown in most of the incidences [reviewed in Bhatnagar et al. (2002)]. For example, at least one of the 10 plagues in ancient Egypt recorded in Exodus (and as early as 430 B.C.) could have been associated with mycotoxin-contamination of food. Since the ninth century, ergot-infected rye has (ergotism) afflicted large-populations in Europe, when ergotism was called *ignis sacer* (sacred fire) or St. Anthony’s fire, because it was believed that a pilgrimage to the shrine of St. Anthony would bring relief from the intense burning sensation caused by the mycotoxin (Van Dongen and DeGroot 1995).

Outbreaks of toxicoses associated with the ingestion of moldy foods and feeds by humans and animals have also been recorded in last century. Deaths of livestock were reported earlier from consumption of moldy corn in feed of horses in Illinois, Russia and swine in Southeastern United States (Christiansen and Kauffman 1969) in the 1930s. A well-documented example is the disease called alimentary toxic aleukia (ATA) that resulted in more than 5000 deaths in humans in the Orenberg district of the USSR during World War II, and the cause of later was found to be trichothecene mycotoxins. Modern mycotoxicology was not developed until the discovery of aflatoxins in the early 1960s as the causative agent in the peanut meal causing the "Turkey X" disease that killed more than 10,000 turkeys fed with the contaminated meal. Because aflatoxins are a series of highly potent carcinogens produced by commonly occurring *Aspergillus flavus* and *A. parasiticus*, research has focused new attention on mycotoxins. In the last 40 years, many new mycotoxins have been identified and characterized, and their biosynthetic origin in various fungi elucidated. It has been estimated that at least 25% of the world's agricultural product is contaminated with mycotoxins and certain diseases have been linked to ingestion of food and feed contaminated with mycotoxins. Recent evidence that indoor air pollution from toxigenic fungi may play a role in illnesses may implicate mycotoxins as having a more widespread role in chronic disease than was previously thought possible (CAST 2003).

1.3 Economic Impact of Mycotoxin Contamination

The most obvious negative economic impact of mycotoxins is an outright loss of crops and affected animals, particularly when a severe outbreak occurs. However, even lower mycotoxin levels in feed affect animal health by causing feed refusal and an increased susceptibility to infection. Crops contaminated with mycotoxins at certain levels cannot be sold for human/animal consumption in countries, which have rigorous regulatory requirement for acceptable level of these compounds. However, humans and animals may encounter severe health hazard or high mortality rates in countries with less regulation or monitoring programs as result of exposure to higher levels of toxins in foods and feeds. In addition, costs of chemical analyses, quality control and regulatory programs, research development, extension services, lawsuits, and the cost of treatment of human illness must be considered in the overall economic burden of mycotoxins on the economy worldwide. Thus, the negative economic impact (crop loss) resulting from mycotoxin contamination is certainly very significant (CAST 2003), and estimated to be \$932 million annually from aflatoxins, fumonisins, and deoxynivalenol contamination of crops.

2 PRODUCTION OF MYCOTOXINS BY TOXICOGENIC FUNGI

2.1 General Consideration

Invasion by fungi and production of mycotoxins in commodities can occur under favorable conditions in the field (preharvest), at harvest, and during processing, transportation and storage (Bhatnagar et al. 2002). Fungi that are frequently found in the field include *A. flavus*, *Alternaria longipes*, *A. alternata*, *Claviceps purpurea*, *Fusarium verticillioides* (previously called *moniliforme*), *F. graminearum*, and a number of other *Fusarium* spp. Species most likely introduced at harvest include *F. sporotrichioides*, *Stachybotrys atra*, *Cladosporium* sp., *Myrothecium verrucaria*, *Trichothecium roseum*, as well as *A. alternata*. Most penicillia are storage fungi. These include *Penicillium citrinum*, *P. cyclopium*, *P. citreoviride*, *P. islandicum*, *P. rubrum*, *P. viridicatum*, *P. urticae*, *P. verruculosum*, *P. palitans*, *P. puberulum*, *P. expansum*, and *P. roqueforti*, all of which are capable of producing mycotoxins in grains and foods. Other toxicogenic storage fungi are: *A. parasiticus*, *A. flavus*, *A. versicolor*, *A. ochraceus*, *A. clavatus*, *A. fumigatus*, *A. rubrum*, *A. chevallieri*, *F. verticillioides*, *F. tricinctum*, *F. nivale*, and several other *Fusarium* spp. It is apparent, most of the mycotoxin-producing fungi belong to three genera: *Aspergillus*, *Fusarium*, and *Penicillium*. However, not all species in these genera are toxicogenic.

2.2 Factors Affecting Mycotoxin Production

Genetics and environmental and nutritional factors greatly affect the formation of mycotoxins. Depending on the susceptibility of the crop, geographic and seasonal factors, as well as cultivation, harvesting, storage, and transportation practices, mycotoxins are found worldwide (D'Mello and MacDonald 1997). In the field, weather conditions, plant stress, invertebrate vectors, species and spore load of infective fungi, variations within plant and fungal species, and microbial competition all significantly affect mycotoxin production. Physical factors such as time of exposure, temperature during exposure, humidity, and extent of insect or other damage to the commodity prior to exposure determine mycotoxin contamination in the field or during storage. Chemical factors including the nutritional status of the crops or chemicals (such as fungicides) used in crop management could affect fungal populations, and consequently toxin production. The temperature and relative humidity range for optimal mycotoxin production may differ from that supporting fungal growth. In general, mycotoxins are optimally produced at 24–28°C, but some toxins such as T-2 toxin is maximally produced at 15°C. Contamination during crop storage may be affected by changes in temperature and

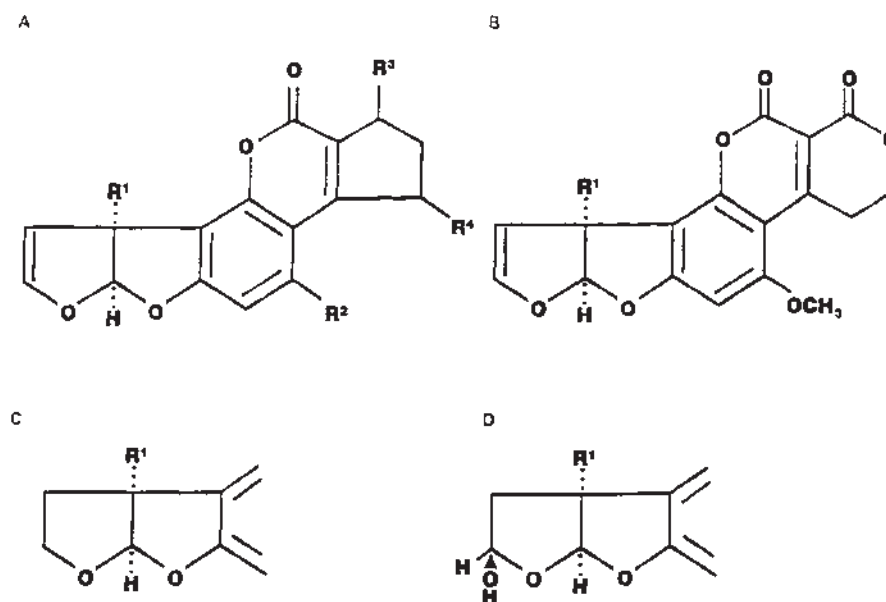
water activity, that allow ecological succession of different fungi as water activity and temperature of stored grain changes. During storage and transportation, water activity (a_w), temperature, crop damage, time, blending with moldy components, and a number of physical and chemical factors, such as aeration (O_2 , CO_2 levels), types of grains, pH, and presence or absence of specific nutrients and inhibitors are important.

3 NATURAL OCCURRENCE AND TOXIC EFFECTS OF SELECTED MYCOTOXINS

3.1 Aflatoxins

3.1.1 General Considerations

Aflatoxins (AF), a group of polyketide-derived bisfurans containing dihydrofuranofuran and tetrahydrofuran (rings)



Aflatoxins	Structure	R ¹	R ²	R ³	R ⁴
B ₁	A	H	OCH ₃	=O	H
M ₁	A	OH	OCH ₃	=O	H
P ₁	A	H	OH	=O	H
Q ₁	A	H	OCH ₃	=O	OH
R ₀	A	H	OCH ₃	OH	H
R ₀ H ₁	A	H	OCH ₃	OH	OH
B ₂	AC	H	OCH ₃	=O	H
B _{2a}	AD	H	OCH ₃	=O	H
M ₂	AC	OH	OCH ₃	=O	H
G ₁	B	H	-	-	-
G ₂	BC	H	-	-	-
G _{2a}	BD	H	-	-	-
GM	BC	OH	-	-	-

Figure 1 Chemical structure of aflatoxins. (A) The B-type aflatoxins are characterized by a cyclopentane E-ring. These compounds have a blue fluorescence under long-wavelength ultraviolet light. (B) The G-type aflatoxins, with a green fluorescence, have a xanthone ring in place of the cyclopentane. (C) Aflatoxins of the B₂ and G₂ type have a saturated bis-furanyl ring. Only the bis-furan is shown. (D) Aflatoxin of the B_{1a} and G_{1a} type have a hydrated bis-furanyl structure.

fused with substituted coumarin (Figure 1) are the most characterized class of mycotoxins (Cole and Cox 1981; Eaton and Groopman 1994). At least 16 structurally related toxins in this group are produced by *A. flavus* and *A. parasiticus* (Bhatnagar et al. 1992; 1993) and infrequently by *A. pseudotamarii* and *A. nominus* (Goto et al. 1996; Ito et al. 2001). *A. ochraceoroseus* has also been found to produce aflatoxins (Klich et al. 2000). The optimal temperatures and a_w for the growth of *A. flavus* and *A. parasiticus* are around 35–37°C (range from 6–54°C) and 0.95 (range from 0.78–1.0), respectively; whereas for aflatoxin production, they are 28–33°C and 0.90–0.95 (0.83–0.97), respectively. Aflatoxin B1 is most toxic in this group and is one of the most potent naturally occurring carcinogens; other AFs are less toxic ($B1 > G1 > B2 > G2$). Other significant members of the aflatoxin family, such as M1 and M2, are metabolites of AFB1 and AFB2, respectively, and originally isolated from bovine milk [reviewed in Cary et al. (2000); Spahr et al. (1999)].

The major steps and the corresponding genes of the AFB1 biosynthesis have been elucidated [extensively reviewed in Bhatnagar et al. (2003)]. Starting with the polyketide precursor, acetate, there are at least 23 enzymatic steps in the AFB1 biosynthetic pathway. AFB2, G1, and G2 are synthesized from pathways that diverge from the B1 pathway. The genes for almost all the enzymes have been cloned and a regulatory gene (*aflR*) coding for a DNA-binding, Gal 4-type 47 kDa protein has been shown to be required for transcriptional activation of all the structural genes. A defect in *aflR* expression in the koji mold *A. sojae* causes to turn-off the expression of AF genes that produce AF (Matsushima et al. 2001). It has also been shown by restriction mapping of cosmid and phage DNA libraries of *A. flavus* and *A. parasiticus* that all the AFB1 pathway genes are clustered within a 75-kb region of the fungal genome (Bhatnagar et al. 2003). G-protein signaling has recently been found to be involved in the biosynthesis of AF (Tag et al. 2000).

3.1.2 Natural Occurrence

Aflatoxins have been found in corn, peanuts and peanut products, cotton seeds, peppers, rice, pistachios, tree nuts (Brazil nuts, almonds, pecans), pumpkin seeds, sunflower seeds and other oil seeds, copra, spices, and dried fruits (figs, raisins), and yams [see CAST (2003) for a detailed list]. Among these products, frequent contamination with high levels of AF in peanuts, corn, and cottonseed, mostly due to infestation with fungi in the field, are of most concern. Soybeans, beans, pulses, cassava, sorghum, millet, wheat, oats, barley, and rice are resistant or only moderately susceptible to AF contamination in the field. It should be reiterated that resistance to AF contamination in the field does not guarantee that the commodities are free of AF contamination during storage. Inadequate storage conditions, such as high moisture and warm temperatures (25–30°C), can create conditions favorable for the growth of fungus and production of AF.

3.1.3 Toxic Effects and Mode of Action

Aflatoxins are mutagenic, teratogenic, and hepatocarcinogenic in experimental animals [reviewed in Eaton and Groopman (1994)]. Since AFB1 is one of the most potent naturally occurring carcinogen, extensive research was primarily done on this toxin. The main target organ of AF is the liver. Typical symptoms of aflatoxicoses in animals include proliferation of the bile duct, centrilobular necrosis, and fatty infiltration of the liver, generalized hepatic lesions, and hepatomas. AFB1 also affects other organs and tissues including the lungs and the entire respiratory system. The susceptibility of animals to AFB1 varies considerably with species in the following order: rabbits (most), ducklings, mink, cats, pigs, trout, dogs, guinea pigs, sheep, monkeys, chickens, rats, mice, and hamsters (least). For the carcinogenic effects, rats, rainbow trout, monkeys, and ducks are most susceptible and mice are relatively resistant. Consumption of AFB1-contaminated feed by dairy cows results in the excretion of AFM1 in milk. AFM1, a hydroxylated metabolite of AFB1, is about 10 times less toxic than AFB1; but its presence in milk is of concern for human health (Chu 2002).

Metabolism plays a key role for the toxic action of aflatoxins. AFB1 must first be activated by mixed-function oxidases to a putative short-lived AFB-8,9-*exo*-epoxide [reviewed in Eaton and Groopman (1994); Sinha and Bhatnagar (1998)]. This intermediate can either be converted to hydroxylated metabolites, conjugated to glutathione or glucuronic acid etc; and then be excreted, or it can bind to DNA, RNA, and protein to exert its carcinogenic, mutagenic, and toxic effects. Glutathione S-transferase serves as a key enzyme in the detoxification process for AFB1. Formation of AF dialdehyde by aldo-keto reductases and subsequent reaction with proteins also plays a role against AFB1 toxicity (Guengerich et al. 2001). Adduct formation of this intermediate with DNA occurs at the N-7 guanine position leading G-C to T-A or to A-T nucleotide substitutions, which results in defective repair and DNA damage, mutations and ultimately carcinomas in many animal species (Chu 2002; Eaton and Gallagher 1994; Wild and Hall 2000; Wogan 2000). Aflatoxin-induced G:C mutations, both G to T and G to A, have been implicated in the inactivation of human *p53* tumor suppressor gene at third position of codon 249; and the identification of mutations/inactivation of *p53* at this site, has been used as a biomarker for AFB-induced liver cancers in humans (Groopman et al. 1995; Harris 1996). Activated AFB1 and AFG1 also formed adducts with albumin (Skipper and Tannenbaum 1990) excreted in urine, which has been used as an index of human exposure to AFs (Sabbioni and Wild 1991; Turner et al. 2000).

3.1.4 Impact on Human Health

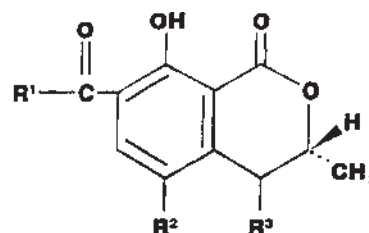
Whereas AFB1 has been found to be a potent carcinogen in many animal species, the role of AF in carcinogenesis in humans is complicated by hepatitis B virus (HBV) infections

in humans (Hsieh 1989; Wild et al. 1992). Epidemiological studies have shown a strong positive correlation between AF levels in the diet and primary hepatocellular carcinoma (PHC) incidence in some parts of the world, including certain regions of the People's Republic of China, Kenya, Mozambique, Philippines, Swaziland, Thailand, and Transkei of South Africa [reviewed in Eaton and Groopman (1994)]. Adducts of AF, i.e., AFB1-DNA and AF-albumin as well as several AF metabolites, mainly AFM1 have been detected in serum, milk, and urine of humans in these regions. However, the prevalence of HBV infection is also correlated to liver cancer incidence in these regions. Since multiple factors are important in carcinogenesis (Wogan 2000) and environmental contaminants such as AFs and other mycotoxins may, either in combination with HBV or independently, be important etiological factors. Recent data on the enhancement of mutation of *p53* gene suggest the synergistic effect of these two risk factors for PHC in humans (Jackson et al. 2001; Smela et al. 2001).

3.2 Ochratoxins

3.2.1 General Considerations

Ochratoxins, a group of dihydroisocoumarin-containing mycotoxins (Figure 2), are produced by a number of fungi in the genera *Aspergillus* and *Penicillium* [reviewed in Bhatnagar et al. 2002; Chu 2002; Ono et al. 1995; Petzinger and Ziegler 2000]. The largest amounts ochratoxins are made by *A. ochraceus* and *P. cyclopium*. *A. ochraceus* and *P. viridicatum* (reclassified as *P. verrucosum*), two species that were first reported as ochratoxin A (OA) producers, occur most frequently in nature. Other fungi, such as *Petromyces alliceus*, *A. citricus*, and *A. fonscaeus* (both in *A. niger* group), have also been found to produce OA. Most of the OA producers are storage fungi and preharvest fungal infection and OA production is not a serious problem. Although most OA producers can grow in a range from 4°C to 37°C and at a_w as low as 0.78, optimal conditions for toxin production are narrower with temperature at 24–25°C and a_w values >0.97 (minimum a_w for OA production is about 0.85). Ochratoxins are produced primarily in cereal grains (barley, oats, rye, corn, wheat) and mixed feed during storage in temperate climatic conditions, with levels higher than 1 ppm being reported. OA has been found in other commodities, including beans, coffee, nuts, olives, raisin, cheese, fish, pork, milk powder, fruit juices wine (Filali et al. 2001; Pietri et al. 2001), beer, peppers (Thirumala et al. 2001), and bread (Van Egmond and Speijers 1994). OA can be carried through the food chain because of the presence of OA residues in animal products as result of its binding with serum albumin. Natural occurrence of OA in kidneys, blood serum, blood sausage, and other sausage made from pork has been reported [reviewed in CAST (2003)].



Ochratoxins	R ¹	R ²	R ³
A	a*	Cl	H
B	a	H	H
C	b	Cl	H
A Methyl ester	c	Cl	H
B Methyl ester	c	H	H
B Ethyl ester	b	H	H
4 - hydroxy ochratoxin A	a	Cl	OH
α	OH	Cl	H
β	OH	H	H

* a = C₆H₅CH₂CH(COOH)NH-

b = C₆H₅CH₂CH(COOEt)NH-

c = C₆H₅CH₂CH(COOMe)NH-

Figure 2 Structure of the ochratoxins. These metabolites form different classes depending on the nature of the amide group (a–c), and the presence or absence of a chlorine moiety at R2 in the phenyl group.

3.2.2 Toxicological Effects and Mode of Action

Ochratoxin A, the most toxic member of this group of mycotoxins, has been found to be a potent nephrotoxin causing kidney damage as well as liver necrosis and enteritis in many animal species [reviewed in Bhatnagar et al. 2002; Chu 2002]. Although OA is primarily considered a weak nephro-carcinogen because a high level of toxin and an extended period of exposure are necessary to induce the tumors, it also causes liver tumors in rats (Boorman et al. 1992; Schlatter et al. 1996). The OA is also an immunosuppressor (Bondy and Pestka 2000; Boorman et al. 1992; Simon 1996) and has teratogenic, mutagenic, weak genotoxic properties (Dirheimer 1996; deGroene et al. 1996).

Several mechanisms have been postulated for the mode of action for OA (Chu 2002). Whereas OA–DNA adducts have been found in kidney, liver, and several other organs of mice and rats, their structures and functions have not been defined (Grosse et al. 1995; Pfohl-Leschkowicz et al. 1993). Formation of a reactive intermediate such as of those for AFB1 is less

likely to be involved in the action of OA (Gautier et al. 2001a, b; Zepnik et al. 2001). The OA inhibits carboxypeptidase A, renal phosphoenolpyruvate carboxykinase, phenylalanine-tRNA synthetase, and phenylalanine hydroxylase activity. Formation of free radicals has been considered as one of the mechanisms for the carcinogenic/toxic effects of OA (Baudrimont et al. 1994; Gautier et al. 2001a; Hoehler and Marquardt 1996; Rahimtula et al. 1988). Studies on the OA induced apoptosis revealed that it interacts with distinct members of the mitogen-activated protein kinase family, e.g., c-jun amino-terminal-kinase, which can lead to direct or indirect caspase 3 activation and subsequently to APT (Gekle et al. 2000; Schwerdt et al. 1999). OA may impair cellular Ca^{2+} cAMP homeostasis. In the renal epithelial cells, OA interferes with hormonal Ca^{2+} signaling, leading to altered cell proliferation.

3.2.3 OA and Human Health

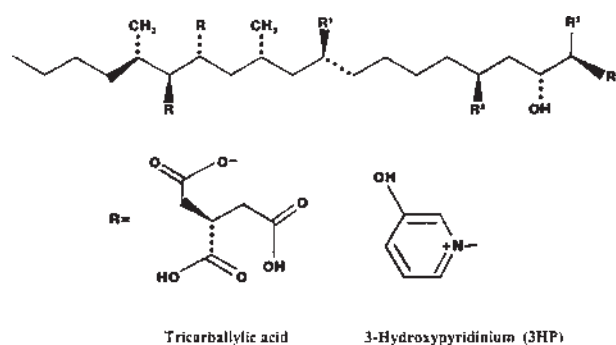
Although the role of ochratoxins in human pathogenesis is still speculative, the pathological lesions of nephropathy in humans were reported to be similar to those observed in porcine nephropathy (Krogh 1976). Outbreaks of kidney disease (Balkan endemic nephropathy) in rural populations in Bulgaria, Romania, Tunisia, and the former Yugoslavia has been associated with OA (Maaroufi et al. 1996; Wolff et al. 2000).

3.3 Fumonisin

3.3.1 General Considerations

Fumonisin (Fm) are a group of toxic metabolites produced primarily by *F. verticillioide*s, *F. proliferatum* and other related species readily colonize corn all over the world (Dutton 1996; Jackson et al. 1996; Marasas 2001). Although *F. anthophilum*, *F. nupiforme*, and *F. nygamai* are capable of producing Fms, they are not commonly isolated from food and feed. Other related fusaria, including *F. subglutinans*, *F. annulatum*, *F. succisae*, and *F. beomiforme*, are not Fm producers (Nelson et al. 1993). More than 11 structurally related Fms (B1, B2, B3, B4, C1, C4, A1, A2, etc.), have been found since the discovery of FmB1 (diester of propane-1,2,3-tricarboxylic acid of 2-amino-12, 16-dimethyl-3,5,10,14,15-pentahydroxyicosane) in 1987 (Figure 3) [reviewed in Bhatnagar et al. (2002); Chu (2002)]. Several hydrolyzed derivatives of Fms, resulting from removal of the tricarballic acid and other ester groups, have also been found in nature. Fumonisin are chemically similar in structure to toxins (AAL) produced by *A. alternata*. Production of fumonisin by *Alternaria* has also been reported, and some fumonisin-producing *Fusaria* have been known to produce AAL toxins.

Fumonisin are most frequently found in corn, corn-based foods, and other grains (such as sorghum and rice) but peanuts and soybeans are poor substrate. The level of contamination varies considerably with different regions and year, ranging



Fumonisin	R ¹	R ²	R ³	R ⁴
B ₁	OH	OH	NH ₂	CH ₃
B ₂	H	OH	NH ₂	CH ₃
B ₃	OH	H	NH ₂	CH ₃
B ₄	H	H	NH ₂	CH ₃
A ₁	OH	OH	NHCOCH ₃	CH ₃
A ₂	H	OH	NHCOCH ₃	CH ₃
C ₁	OH	OH	H	H
C ₄	H	H	H	H
P ₁	OH	OH	3HP	CH ₃
P ₂	H	OH	3HP	CH ₃
P ₃	OH	H	3HP	CH ₃

Figure 3 Chemical structures of different classes of fumonisin. The R residue is 3-hydroxypyridinium in fumonisin, B1–B3 while the others are tricarballic esters.

from negligible to more than 100 ppm; but is generally reported to be between 1 and 2 ppm. FmB1 is the most common Fm in naturally contaminated samples; FmB2 generally accounts for 1/3 or less of the total. Although production of the toxin generally occurs in the field, continued production of toxin during postharvest storage also contributes to the overall levels. (Chu 2001; 2002; 2001 May issues of Environmental Health Prospective, Vol. 109).

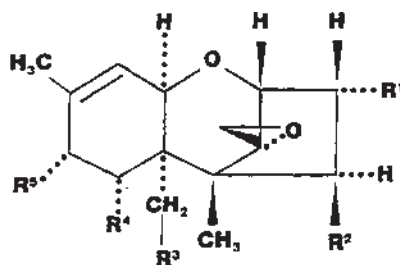
The biosynthesis of the 20-carbon chain backbone of Fms resembles those for fatty acids and linear polyketides [reviewed in Desjardins et al. 1996; Proctor 2000]. Five loci (designated as fum 1, fum 2, fum 3, and fum 4 and fum 5) have been identified by classical genetic analyses utilizing *Gibberella fujikuroi*, the sexual stage of *F. moniforme* (Proctor 2000). At least 15 genes are associated with the fumonisin production and these genes are clustered together on chromosome “1” (Proctor 2000; Xu and Leslie 1996) including a polyketide synthase gene (*fum5*). Gene *Fum2* and *Fum3* are associated with the interconversion of different fumonisin (Proctor 2000).

3.3.2 Toxicologic Effects

Fumonisin B1 is primarily a hepatotoxin and carcinogen in rats (Class 2B carcinogen). Feeding culture material from

F. verticillioides or pure FmB1 to rats resulted in cirrhosis and hepatic nodules, adenofibrosis, hepatocellular carcinoma-ductular carcinoma, and cholangiocarcinoma (Gelderblom et al. 2001; Haschek et al. 2001). Kidney is also a target organ, and tubular nephrosis was found both in rats and in horses of field cases associated with equine leukoencephalomalacia (ELEM). In addition to FmB1, which was originally found to be a potent cancer-causing agent, FmB2 and FmB3 have also been found to be carcinogens and have cancer initiation and promoting activities in rats. The effective dose of FmB1 for cancer initiation in rat liver depends both on the levels and on the duration of exposure. In cell culture systems, FmB1 has been demonstrated to be mitogenic and cytotoxic, without genotoxic effects (Gelderblom et al. 2001). Kidney cells have also been shown to be targeted by these toxins.

Fumonisin B1 was identified as an etiological agent responsible for ELEM in horses and other Equidae (donkeys and ponies) and for porcine pulmonary edema (PPE) [Jackson et al. 1996; reviewed in Summerell et al. (2001)]. ELEM is characterized primarily by neurotoxic effects, including uncoordinated movements and apparent blindness showing as violent blundering into stalls and walls. The levels of FmB1 and FmB2 in feeds associated with confirmed cases of ELEM ranged from 1.3 to 27 ppm. In pigs, PPE occurs only at high FmB levels (175 ppm), while liver damage occurs at much lower concentrations with a NOAEL of <12 ppm. FmB1 resulting in left-sided heart failure alters cardiovascular function. In the cattle, renal injury, hepatic lesions, and alteration of sphingolipid in various organs was observed (Haschek et al. 2001). Similar to AAL toxin, Fms are also



Trichothecenes	R ¹	R ²	R ³	R ⁴	R ⁵
Diacetoxyscirpenol	OH	OAc ¹	OAc	H	H
4-Monoacetoxyscirpenol	OH	OAc	OH	H	H
15-Monoacetoxyscirpenol ^a	OH	OH	OAc	H	H
Scirpentriol	OH	OH	OH	H	H
Deoxynivalenol	OH	H	OH	OH	=O
Nivalenol	OH	OH	OH	OH	=O
Fusarenon-x	OH	OAc	OH	OH	=O
T-2	OH	OAc	OAc	H	ISV ²
HT-2	OH	OH	OAc	H	ISV
T-2 triol	OH	OH	OH	H	ISV
3'-OH-T-2	OH	OAc	OAc	H	OH-ISV ³
T-2 tetraol	OH	OH	OH	H	OH
Neosolaniol	OH	OAc	OAc	H	OH
Roridin A,B,E,H,J	H	MC ^a	MC	H	H
Roridin K	H	MC	MC	H	OH
Verrucarins A,B,J,K	H	MC	MC	H	H
Verrucarins L	H	MC	MC	H	OH
Satratoxins F,G,H	H	MC	MC	H	H
Verrucarol	H	OH	OH	H	H

^a Macrocyclic

¹ OAc OCOCH₃

² ISV OCOCH₂CH(CH₃)₂

³ OH-ISV OCOCH₂C(OH)(CH₃)₂

Figure 4 Chemical structure of different trichothecenes. MC = Macrocyclic; ISV = isovalerate; OH-ISV = hydroxyisovalerate.

toxic to some plants such as, jimsonweed, black nightshade, duckweed, and tomatoes (Abbas and Riley 1996).

3.3.3 Mode of Action

Mechanistically, Fms are inhibitors of ceramide synthase (sphinganine/sphingosine N-acyltransferase), a key enzyme involved in the biosynthesis of sphingolipids, which are heavily involved in cellular regulation, including cell differentiation, mitogenesis and apoptosis (Merrill et al. 2001; Riley et al. 2001). The primary amino group of Fms is essential for its inhibitory and toxic effects (Norred et al. 2001). The inhibitory effect of Fms on ceramide synthase appears to be related to interference with sphingolipid biosynthesis in multiple organs, such as brain, lung, liver, and kidney of the susceptible animals (Garren et al. 2001). Increased lipid peroxidation has also been considered as a mode for its effect on initiation of cancer (Gelderblom et al. 2001). The mode of action of FmB becomes more clear from recent data of its effects on apoptosis (Zhang et al. 2001) and the tumor necrosis factor (TNF) pathway is important in inducing tumors (He et al. 2001; Zhang et al. 2001; Sharma et al. 2001), but the tumor suppressor gene p53 was not required (Jones et al. 2001). The ability of FmB1 to alter gene expression and signal transduction pathways are considered necessary for its carcinogenic and toxic effects. FmB1 is a good example of an apparently nongenotoxic (non-DNA reactive) agent producing tumors through the regulation of apoptosis (Dragan et al. 2001).

3.3.4 Impact on Human and Animal Health

While Fms are commonly detected in corn-based foods and feeds, the impact of low levels of Fms in human foods is not clear. Although several reports have indicated a possible role of FmB1 in the etiology of human esophageal cancer in the regions of South Africa, China, and northeastern Italy where *Fusarium* species are common contaminants, more data are necessary to sustain this hypothesis (Groves et al. 1999). The co-occurrence of Fms with carcinogenic mycotoxins such as AFB (da-Silva et al. 2000; Vargas et al. 2001) or nitrosamines may play an important role in carcinogenesis in humans. Current data suggest that Fms may have greater effect on the health of farm animals than on humans [Bhatnagar et al. (2002) for review].

3.4 Selected Trichothecenes (TCTCs)

3.4.1 General Considerations

Several species of *Fusaria* infect corn, wheat, barley, and rice. Under favorable conditions, they elaborate a number of different types of tetracyclic sesquiterpenoid mycotoxins that are composed of the epoxytrichothecene skeleton and an olefinic bond with different side chain substitutions (Figure 4). Based on the presence of a macrocyclic ester or ester-ether bridge between C-4 and C-15, trichothecenes (TCTCs) are

generally classified as macrocyclic (Type C) or nonmacrocyclic (Types A and B) (Figure 4). Although more than 100 TCTCs have been identified, only a few frequently found in foods and feeds are potentially hazardous to human and animal health. Other fungal genera are *Myrothecium*, *Trichoderma*, *Trichothecium*, *Cephalosporium*, *Verticimonosporium*, and *Stachybotrys*. In addition to fungi, extracts from a Brazilian shrub, *Baccharis megapotamica*, also contain macrocyclic TCTCs. The term TCTCs is derived from trichothecin, the first compound isolated in this group (Chu 1997; Jarvis et al. 1995; Miller and Trenholm 1994; Vesonder and Golinski 1989).

Similar to other sesquiterpenes, TCTCs are biosynthesized via the mevalonate pathway: the TCTC skeleton is formed by cyclization of farnesyl pyrophosphate via the intermediate trichodiene by an enzyme trichodiene synthase. The isovaleroxy side chain in T-2 toxin is derived from leucine. Several key enzymes in the TCTC biosynthetic pathway have been identified. At least six genes (*Tri1* to *Tri6*) involved in the biosynthesis of these mycotoxins have been cloned, and these genes are clustered together on a chromosome. Genes *Tri3*, *Tri4*, and *Tri5*, which encode a transacetylase (15-O-acetyltransferase), a cytochrome P-450 monooxygenase, and trichodiene synthase, respectively, are contained within a 9-kb region, while *Tri5* and *Tri6* (a regulatory gene) are in a 5.7 kb region (Proctor 2000).

The structural diversity of TCTCs results in a variety of toxic effects in animals and humans [reviewed in Bhatnagar et al. (2002)]. The TCTC mycotoxins affect many organs, including the gastrointestinal tract, hematopoietic, nervous, immune, hepatobiliary, and cardiovascular systems. Outbreaks of several types of mycotoxicoses in humans and animals, including moldy corn toxicosis, scabby wheat toxicosis (or red-mold, or akakbi-byo disease, or scabby barley poisoning), feed refusal and emetic syndrome (swine), fusaritoxicoses, hemorrhagic syndrome, and alimentary toxic aleukia (ATA) have been reported. Mechanistically, inhibition of protein synthesis is one of the earlier events in manifestation of TCTC toxic effects and they act at different steps in the translation process. Inhibitory effects of these mycotoxins vary considerably with the chemical structure of the side chain [see review in Chu (2002)].

3.4.2 T-2 Toxin and Related Type A TCTCs

T-2 toxin, a highly toxic type A TCTC, is produced by *F. tricinctum*, *F. sporotrichioides* (major), *F. poae*, *F. sulphureum*, *F. acuminatum*, and *F. sambucinum*. Unlike most mycotoxins, which are usually synthesized near 25°C, the optimal temperature for T-2 toxin production is around 15°C. Higher temperatures (20–25°C) are needed for the production of related metabolites, such as H-T2 toxin. Although T-2 toxin occurs naturally in cereal grains, including barley, corn, corn stalk, oats, wheat, and mixed feeds, contamination with T-2 toxin is less frequent than with deoxynivalenol (DON). However, T-2 toxin (LD₅₀ in

mice: 2–4 mg/kg mice) is much more toxic to animals, perhaps also to humans, than DON (LD₅₀ in mice: 50–70 mg/kg).

Almost all the major TCTCs, including T-2 toxin, are cytotoxic and cause hemorrhage, edema, and necrosis of skin tissues. Inflammatory reactions near the nose and mouth of animals are similar to some lesions found in humans suffering from ATA disease. The severity of lesions is also related to chemical structure. Compared with other types of TCTCs, group A toxins (T-2 toxin) are less toxic than macrocyclic toxins but more toxic than type B toxins. Neurologic dysfunctions, including emesis, tachycardia, diarrhea, refusal of feed/anorexia, and depression have also been observed. T-2 toxin and some TCTCs also induce pathological lesions in the gastrointestinal system. However, the major lesion of T-2 toxin is its devastating effect on the hematopoietic system in many mammals, including humans. T-2 toxin and related TCTCs are the most potent immunosuppressants of the known mycotoxins and cause significant lesions in lymph nodes, spleens, thymus, and the bursa of Fabricius (Bondy and Pestka 2000). The heart and pancreas are other target organs for T-2 toxin intoxication. Urinary and hepatobiliary lesions induced by T-2 toxin and DAS are secondary.

3.4.3 Deoxynivalenol (DON)

The DON is a major type B TCTC mycotoxin produced by *F. graminearum* (major) and other related fungi such as *F. culmorum* and *F. crookwellense*. Because DON causes feed refusal and emesis in swine, the name “vomitoxin” is also used for this mycotoxin. Although DON is considerably less toxic than most other TCTC mycotoxins, the level of contamination of this toxin in corn and wheat is generally high, usually above 1 ppm, sometimes greater than 20 ppm. Contamination of DON in other commodities, including barley, oats, sorghum, rye, safflower seeds, and mixed feeds has also been reported. Worldwide frequent natural occurrence of DON in cereal grains has been reported (Miller and Trenholm 1994). Although inadequate storage may lead to the production of some TCTC mycotoxins, infestation of fusaria in wheat and corn in the field is of most concern for the DON problem. With wet and cold weather during maturation, grains are especially susceptible to *F. graminearum* infection, which causes so-called “scabby wheat” and simultaneously produces the toxin. The optimal temperature for DON production is about 24°C. Outbreaks of DON in winter wheat in the United States, Finland, and Canada usually occur when continental chilly and humid weather favoring the fungal infection is followed by a humid summer favorable for toxin production.

Toxicologically, DON induces anorexia and emesis both in humans and animals [reviewed in Bhatnagar et al. (2002); Chu (2002)]. Swine are most sensitive to feed contaminated with DON. Whereas most TCTCs are immunosuppressors, DON is a hyperinducer of cytokines and IgA. Induction of expression of mRNA of *ILs*-2, 4, 5, 6 in a T-cell model EL4.IL-2 by DON was found at levels required for partial or maximal protein synthesis inhibition. A single oral gavage

with DON was sufficient to induce these mRNA levels in Peyer’s patches and spleen (Bondy and Pestka 2000).

3.4.4 The Impact of TCTC on Human and Animal Health

Because of their toxicity and their frequent presence in foods and feeds, TCTCs are potentially hazardous to human and animal health. Among the many types of TCTC mycotoxins, only ATA and scabby wheat toxicosis have been demonstrated in human populations. ATA, which has symptoms including skin inflammation, vomiting, damage to hematopoietic tissues, leukocytosis, and leukopenia, was attributed to the consumption of overwintered cereal grains colonized by *F. sporotrichioides* and *F. poae*; it caused the deaths of hundreds of people in the USSR between 1942 and 1947 (Ueno 1986). TCTCs may also be involved in the so-called “sick building syndrome (SBS)” in humans (Mahmoudi and Gershwin 2000; Vesper et al. 2000). *S. atra* and *S. chartarum* were isolated from a badly water-damaged home or building where the occupants complained about headaches, sore throats, hair loss, flu symptoms, diarrhea, fatigue, dermatitis, and general malaise as well as pulmonary hemorrhage cases. Several TCTCs were found in these cases, but other molds and mycotoxins were also found in the mold damaged buildings (Toumi et al. 1993).

3.5 Other Selected Mycotoxins

In addition to the mycotoxins discussed above, a number of other mycotoxins occur naturally. The impacts of some of these mycotoxins on human and animal health are discussed in the following sections.

3.5.1 Other Mycotoxins Produced by *Aspergillus*

Sterigmatocystin (ST) is a naturally occurring hepatotoxic and carcinogenic mycotoxin produced by fungi in the genera *Aspergillus*, *Bipolaris*, and *Chaetomium* as well as *P. luteum* [see Bhatnagar et al. (2002)]. Structurally related to AFB1 (Figure 5), ST is known to be a precursor of AFB1 (Bhatnagar

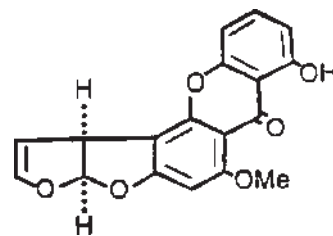


Figure 5 Structure of sterigmatocystin. The bis-furanyl structure is similar to that of the aflatoxins except that the E-ring is a substituted phenol.

et al. 2003). Although the carcinogenicity of ST is less (10–100 times) than that of AFB1 in test animals (van der Watt 1977), ST is a mutagen and genotoxin and has been found in cereal grains (barley, rice, and corn), coffee beans, and cheese (Chu 2002). *A. terreus* and several other fungi (e.g., *A. flavus* and *A. fumigatus* and some *Penicillia*) have been found to produce the tremorgenic toxins, territrem, aflatrem, and fumitremorgin. These mycotoxins contain both the indole ring of tryptophan and a dioxopiperazine ring formed by condensation of two amino acids. *A. terreus*, *A. fumigatus*, and *Trichoderma viride* also produce gliotoxin, an epipolythiopiperazines -3,6-diones-sulfur containing piperazine antibiotic, that may have immunosuppressive effects in animals (Waring and Beaver 1996). In addition, *A. flavus*, *A. wentii*, *A. oryzae*, and *P. atraovenetum* are capable of producing nitropropionic acid (NPA), a mycotoxin causing apnea, convulsions, congestion in lungs and subcutaneous vessels, and liver damage in test animals (Burdock et al. 2001). Production of NPA in sugarcanes by *Arthrinium sacchari*, *Arth. saccharicola*, and *Arth. phaeospermum* has been found to be involved in fatal food poisoning in humans (Liu et al. 1988).

3.5.2 Other Mycotoxins Produced by *Penicillium*

Other than OA, *Penicillia* produce many mycotoxins with diverse toxic effects. Cyclochlorotine, luteoskyrin (LS), and rugulosin (RS) have long been considered to be possibly involved in the yellow rice disease during the Second World War. They are hepatotoxins and also produce hepatomas in test animals. However, incidents of food contamination with these toxins have not been well documented. Several other mycotoxins, including patulin (PT, Figure 6), penicillic acid (PA, Figure 7), citrinin (CT), cyclopiazonic acid (CPA, Figure 8), citreoviridin, and xanthomegnin, which are produced primarily by several species of *Penicillia*, have attracted some attention because of their frequent occurrence in foods. PT and PA are produced by many species in the genera *Aspergillus* and *Penicillium*. *Byssosclamyces nivea* also produces PT (Tournas 1994). Both toxins are hepatotoxic and

teratogenic. Patulin is frequently found in damaged apples, apple juice, apple cider and sometimes in other fruit juices and feed. PA has been detected in “blue eye corn” beans and meat. Due to its highly reactive double bonds that readily react with sulfhydryl groups in foods, patulin is not very stable in foods containing these groups (Scott 1975). As a hepatotoxin but not known as a carcinogen, PT is considered a health hazard to humans (CAST 2003).

Frequently associated with the natural occurrence of OA, citrinin, also a nephrotoxin, is produced by *P. citrinum* and several other *penicillia*, *aspergilli* (Cole and Cox 1981) and *Monococcus ruber* and *M. purpureus* (Pastrana et al. 1996). The presence of citrinin in the diet with low quality corn could lead to chronic, hard to diagnose kidney disease in susceptible individuals and animals (CAST 2003). One of the mycotoxins closely associated with the natural occurrence of AF in peanuts is CPA, which causes hyperesthesia and convulsions as well as liver, spleen, pancreas, kidney, salivary gland, and myocardial damage (CAST 2003). CPA inhibits the calcium-dependent ATPase (Chu 2002; Petr et al. 1999). The toxin is produced by several species of the genus *Penicillium*, including *P. cyclopium*, *P. crustosum*, *P. griseofulvin*, *P. puberulum*, *P. camemberti*, and *Aspergilli* including *A. versicolor*, *A. flavus* but not by *A. parasiticus* and *A. tamarii* [in Bhatnagar et al. (2002)]. Other than peanuts, CPA has been found in corn, cheese and fermented meat sausage, and sometimes along with aflatoxin (Fernandez-Pinto et al. 2001; Lopez-Diaz et al. 2001).

Penicillium rubrum and *P. purpurogenum* produce two highly toxic hepatotoxins (LD₅₀, 3.0 mg/kg mice, IP) called rubratoxins A (RA, minor) and B (RB, major), which are complex nonadrides fused with anhydrides and lactone rings. Rubratoxin B has synergistic effects with AFB1 (CAST 2003). In addition, *penicillia* produce many neurotoxic mycotoxins. In many cases these mycotoxins do not cause noticeable toxicity, but in some cases have strong tremorgenic activity. Animals will also refuse food, and will have lowered resistance to disease. For example, *P. crustosum* and *P. cyclopium* produce tremorgenic indoleterpenes called penitrem A-F. Penitrem A, the major toxin in this group, causes tremorgenic effects in mice. Roquefortines A-C (C is

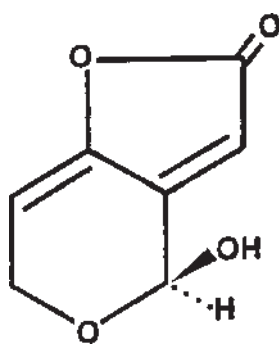


Figure 6 Structure of patulin showing the closed-ring tautomeric form.

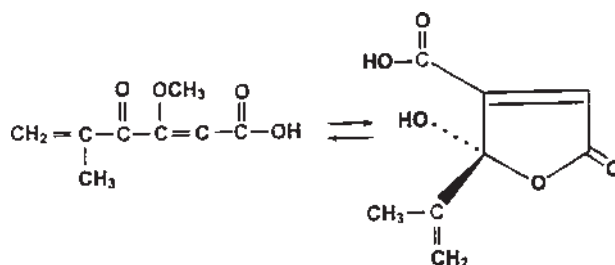


Figure 7 Chemical structure of penicillic acid showing both tautomeric forms. The open-chain form is in equilibrium with the closed form shown on the right in the figure.

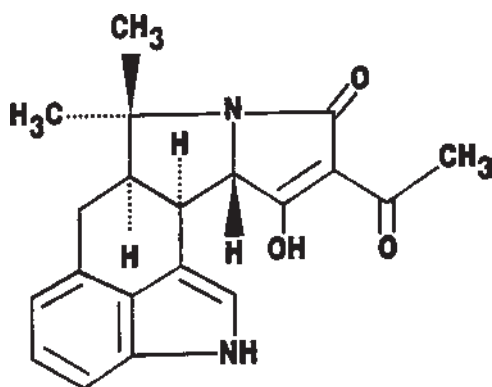


Figure 8 Chemical structure of cyclopiazonic acid.

most toxic), which are produced by *P. roqueforti* and several other penicillia, have neurotoxic effects in animals and have been found in cheese. Tremorgens in the paspalitrem group (paspalicine, paspalinine, paspalitrem A and B, paspaline and paxilline) are produced by *C. paspali* and some penicillia (Plumlee and Galey 1994; Steyn 1995; Yamaguchi et al. 1993).

3.5.3 Other Mycotoxins Produced by *Fusarium*

Some fusaria are capable of producing mycotoxins other than TCTCs and Fm. Zearalenone (ZE) (Figure 9) [6-(10-hydroxy-6-oxo-trans-1-undecenyl)- β (beta)-resorcylic acid lactone], a mycotoxin produced by the scabby wheat fungus, *F. graminearum* (roseum), is of most concern. Also called F-2, ZE is a phytoestrogen causing hyperestrogenic effects and reproductive problems such as premature onset of puberty in female animals, especially swine. ZE has been shown to bind with the estrogen and steroid receptors, and stimulates protein synthesis by mimicking hormonal action (Zepnik et al. 2001). Zearalenone can be toxic to plants; it can inhibit seed germination and embryo growth at low concentrations. Natural contamination with ZE primarily occurs in cereal grains such as corn and wheat. Contamination of feed with ZE

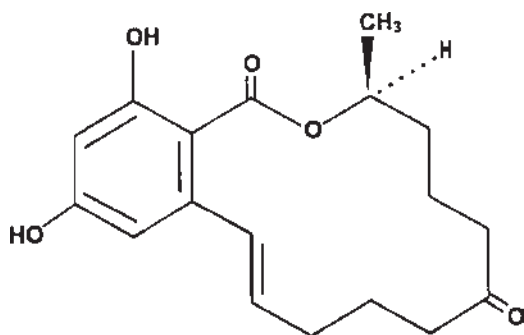


Figure 9 Structure of zearalenone.

in conjunction with DON may result in severe economic losses to the swine industry.

Fusarium verticillioides and related species, in addition to Fms, also produce several other mycotoxins, including fusarins A-F, moniliformin, fusarioic, and fusaric acid, fusaproliferin and beauvericin (CAST 2003; Chu 2002). Although the impact of these mycotoxins on human health is still not known, fusarin C (FC) has been identified as a potent mutagen and is also produced by *F. subglutinans*, *F. graminearum* and several other Fusaria. Moniliformin, which causes cardiomyopathy in test animals, may be involved in the Keshan disease in humans in regions where dietary selenium deficiency is also a problem (Liu 1996). In comparing their ability to form DNA-adducts, beauvericin forms a more stable complex with DNA than fusaproliferin (Pocsfalvi et al. 2000). Among many fungi, *F. verticillioides* is also most capable of reducing nitrates to form potent carcinogenic nitrosamines. These observations further suggest that the contamination of foods with this fungus could be one of the etiological factors involved in human carcinogenesis in certain regions of the world.

3.5.4 Mycotoxins Produced by *Alternaria* Species

Alternaria has been known for centuries to cause various plant diseases. Species of this fungus are widely distributed in soil and on aerial plant parts. More than 20 species of *Alternaria* are known to produce about 70 secondary metabolites belonging to a diverse chemical group, including dibenzo- α -pyrones, tetramic acids, lactones, quinones, cyclic peptides. However, only alternariol (Figure 10), tenuazonic acid, altertoxin-I, alternariol monomethyl ether (AME), altenuene are common contaminants in consumable items like fruits (apples), vegetables (tomato), cereals (sorghum, barely, oat), and other plant parts (such as leaves) (Jelinek et al. 1989). Natural occurrence of isoaltenuene, altenuisol, altertoxins II and III (also called stemphytoxin) are less common. The most common species of *Alternaria*, *A. alternata* (formerly known as *A. kikuchiana*) produces all important *Alternaria* toxins including the five mentioned above and tentoxin, alteniusol, alternaric acid, altenusin, dehydroaltenuisin (Bottalico and Logrieco 1998; Chelkowski

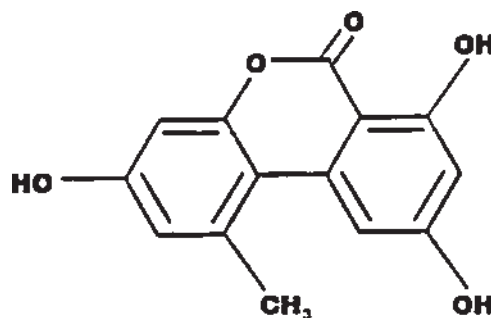


Figure 10 Structure of alternariol.

1992). As mentioned previously, *A. alternata* f. sp. *lycopersici* produces a group of host-specific toxins named AAL toxins with structure and functions similar to Fms. Although most of the compounds produced by *Alternaria* are generally nontoxic, AME has been shown to be mutagenic in Ames test (Woody and Chu 1992). Tenuazonic acid is a protein synthesis inhibitor and is capable of chelating metal ions and forming nitrosamines. This mycotoxin is also produced by *Phoma sorghina* and *Pyricularia oryzae* and may be related to "Onyalai," a hematological disorder in humans living south of the Sahara in Africa.

3.5.5 Mycotoxins Produced by Other Fungi

Sporidesmines, a group of hepatotoxins discovered in the 1960s, are also worthy of mention. These mycotoxins, causing facial eczema in animals, are produced by *Pithomyces chartarum* and *Sporidesmium chartarum* and are very important economically to the sheep industry. Slaframine, a significant mycotoxin produced by *Rhizoctonia leguminicola* (in infested legume forage crops), causes excessive salivation or slobbering in ruminants as a result of blocking acetylcholine receptor sites (CAST 2003).

4 PREVENTIVE MEASURES

4.1 Management of Mycotoxin Contamination

The economic implications of the mycotoxin problem and its potential health threat to humans have clearly created a need to eliminate or at least minimize mycotoxin contamination of food and feed. While an association between mycotoxin contamination and inadequate storage conditions has long been recognized, studies have revealed that seeds are contaminated with mycotoxins prior to harvest (Lisker and Lillehoj 1991). Therefore, management of mycotoxin contamination in commodities must include both pre- and postharvest control measures (CAST 2003).

4.1.1 Preharvest Control

Mycotoxin contamination can be reduced somewhat by using of resistant varieties (most effective, but not all are successful) and earlier harvest varieties, crop rotation, adequate irrigation, control of insect pests, etc [reviewed in Bacon et al. (2001); Chen et al. (2002); Duvick (2001); Sinha and Bhatnagar (1998)]. Significant control of toxin contamination is expected to be dependent on a detailed understanding of the physiological and environmental factors that affect the biosynthesis of the toxin, the biology and ecology of the fungus, and the parameters of the host plant–fungal interactions. Efforts are underway to study these parameters primarily for the most agriculturally significant toxins, namely AFs, Fms, and TCTCs [reviewed in Brown et al. (1998); Duvick (2001); Sinha and Bhatnagar (1988)].

Use of atoxigenic biocompetitive, native *A. flavus* strains to out-compete the toxigenic isolates has been effective in significantly reducing preharvest contamination with aflatoxin in cotton and peanuts (Cotty and Bhatnagar 1994; Dorner et al. 1992). However, the aflatoxin contamination process is so complex (Payne 1998) that a combination of approaches will be required to eliminate or even control the preharvest toxin contamination problem (Bhatnagar et al. 1995).

4.1.2 Postharvest Control

After harvest, crop should not be allowed to overwinter in the field as well as subjected to bird and insect damage or mechanical damage. Grains should be cleaned and dried quickly to less than 10–13% moisture and stored in a clean area to avoid insect and rodent infestation (Trenholm et al. 1988). Postharvest mycotoxin contamination is prevalent in most tropical countries due to a hot, wet climate coupled with subadequate methods of harvesting, handling, and storage practices, which often lead to severe fungal growth and mycotoxin contamination of food and feed (Birzele et al. 2000; Phillips et al. 1994). Sometimes contaminated food has been diverted to animal feed to prevent economic losses and health concerns. However, this is not a solution to the contamination problem. Irradiation has been suggested as a possible means of controlling insect and microbial populations in stored food, and consequently, reducing the hazard of mycotoxin production under these conditions [reviewed in Sharma (1998)]. Significant emphasis has been placed on detoxification methods to eliminate the toxins from the contaminated lots or at least reduce the toxin hazards by bringing down the mycotoxin levels under the acceptable limits.

a. Removal or Elimination of Mycotoxins. Since most of the mycotoxin burden in contaminated commodities is localized to a relatively small number of seeds or kernels [reviewed in Dickens (1977)], removal of these contaminated seeds/kernels is effective in detoxifying the commodity. Methods currently used include: (a) physical separation by identification and removal of damaged seed; mechanical or electronic sorting; flotation and density separation of damaged or contaminated seed; physical screening and subsequent removal of damaged kernels by air blowing; washing with water or use of specific gravity methods have shown some effect for some mycotoxins, including DON, FmB, and AFB1 (Trenholm et al. 1992), (b) removal by filtration and adsorption onto filter pads, clays, activated charcoal, etc., (c) removal of the toxin by milling processes, (d) removal of the mycotoxin by solvent extraction (CAST 2003; DeVries et al. 2002).

b. Inactivation of Mycotoxins. When removal or elimination of mycotoxins is not possible, mycotoxins can be inactivated by (a) physical methods such as thermal inactivation, photochemical or gamma irradiation, (b)

chemical methods such as a treatment of commodities with acids, alkalis, aldehydes, oxidizing agents, and gases like chlorine, sulfur dioxide, NaNO_2 , ozone and ammonia, (c) biological methods such as fermentations and enzymatic digestion that cause the breakdown of mycotoxins. The commercial application of some of these detoxifying mechanisms is not feasible because, in a number of cases, the methods will be limited by factors such as the toxicity of the detoxifying agent, nutritional or aesthetic losses of commodities during treatment, and the cost of the sophisticated treatment [reviewed in Sinha and Bhatnagar 1998]. Although several detoxification methods have been established for aflatoxins, only the ammoniation process is an effective and practical method (Piva et al. 1995). Other chemicals such as ozone, chlorine, and bisulfite have been tested and some effect for some mycotoxins was shown in it (Doyle et al. 1982). Solvent extractions have been shown to be effective but are not economically feasible.

c. Removal of Mycotoxins During Food Processing. While cooking generally does not destroy mycotoxins, some mycotoxins can be detoxified or removed by certain kinds of food processing. For example, extrusion cooking appears to be effective for detoxifying DON but not AFB. FmB1 can form Schiff's bases with reducing sugars such as fructose under certain conditions (Murphy et al. 1995) and lose its hepato-carcinogenicity (Liu et al. 2001); but the hydrolyzed FmB1 was found to be still toxic (Voss et al. 1996). Loss of FmB1 occurs during extrusion and baking of corn-based foods with sugars and nixtamalization (alkaline cooking) and rinsing in the preparation of tortilla chip and masa (Dombrink-Kurtzman et al. 2000; Voss et al. 2001). PT can be removed from apple juice by treatment with certain types of active carbons (Leggott et al. 2001). The effect of food processing on various mycotoxins has been recently reviewed by several authors in an ACS symposium (DeVries et al. 2002).

4.2 Avoiding Human Exposure

4.2.1 Role of Rigorous Monitoring Programs

While it is impossible to remove mycotoxins completely from foods and feeds, effective measures to decrease the risk of exposure depend on a rigorous program of monitoring mycotoxins in foods and feeds. Consequently, governments in many countries have set limits for permissible levels or tolerance levels for a number of mycotoxins in foods and feeds. Over 50 countries of the world have developed such guidelines (Van Egmond 2002). For example, levels varying from zero tolerance to 50 ppb have been set for total AFs. Other than AFs, a tolerance level of 1 ppm for DON in grains for human consumption has been set by a number of countries, including the United States. The FmB1 levels established by FDA in 2000 are limited to 5, 20, 60, 100, 30, and 10 ppm, in corn and corn by-products to be used for horse and rabbit, catfish and swine, ruminants and mink, poultry,

breeding stock (ruminant, poultry, and mink), and others (dogs and cats), respectively. Among 77 countries which have regulations for different mycotoxins, eight have specific regulations for OA, with limits ranging from 1 to 20 $\mu\text{g/kg}$ in different foods. Regulatory guidelines to limit the presence of PT to 50 $\mu\text{g/kg}$ in various foods and juices have been established by at least ten countries worldwide. Details on worldwide regulatory issues and permissive levels of mycotoxins in foods and feeds have appeared in a number of recent reviews (Park and Troxell 2002; Van Egmond 2002; Chu 2002).

4.2.2 Detection and Screening of Mycotoxins

Because of the diverse chemical structures of mycotoxins, the presence of trace amounts of toxins in very complicated matrices that interfere with analysis, and the uneven distribution of the toxins in the sample, analysis of mycotoxins is a difficult task (Chu 1995; Coker 1998; Richard et al. 1993; Wilson et al. 1998). Because many steps are involved in the analysis, it is not uncommon that the analytical error can amount to 20–30% (Horwitz et al. 1993). To obtain reliable analytical data, an adequate sampling program and an accurate analytical method are both important (Whitaker et al. 1994; 1995; Wilson et al. 1998). To minimize the errors, studies have led to many improved and innovative analytical methods for mycotoxin analysis over the years (Chu 1991; Gilbert 1999; Maragos 1997). New, more sensitive TLC, HPLC, and GC techniques are now available. Sensitive and versatile high resolution MS and GC/tandem MS/MS are coming to the market. The MS methods have also been incorporated into HPLC systems. New chemical methods, including capillary electrophoresis and biosensors are emerging and have gained application for mycotoxin analysis (Maragos 1997).

After a number of years of research, immunoassays have gained wide acceptance as analytical tools for mycotoxins in the last decade (Chu 2001). Antibodies against almost all the mycotoxins are now available. Some quantitative and qualitative immunoassays have been approved as AOAC methods. Many immunoscreening kits, which require less than 15 min. per test, are commercially available (Trucksess and Wood 1997). Rather than analysis of toxin, PCR methods, based on the primers of key enzymes involved in the biosynthesis of mycotoxins, have been introduced for the determination of toxicogenic fungi present in foods (Birzele et al. 2000; Edwards et al. 2001). Detailed protocols for mycotoxin analysis can be seen in several of the most recent reviews and books and the most recent edition of AOAC (Gilbert 1999; Trucksess and Pohland 2001; Van Dolah and Richard 1999).

4.2.3 Dietary Modifications

Dietary modification greatly affects the absorption, distribution, and metabolism of mycotoxin and subsequently affect its toxicity [reviewed in Bhatnagar et al. (2002)]. For

example, the carcinogenic effect of AFB1 is affected by nutritional factors, dietary additives, and anticarcinogenic substances. Diet containing chemoprotective agents and antioxidants such as ascorbic acid, BHA, BHT, ethoxyquin, oltipraz, penta-acetyl geniposide, Kolaviron biflavonoids, and even green tea, have also been found to inhibit carcinogenesis caused by AFB1 in test animals. The toxic effect of OA and FmB to test animals was minimized when antioxidants such as vitamins C and E are added to the diet. Ascorbic acid also provided protective effect against AFs. Aspartame, which is partially effective in decreasing the nephrotoxic and genotoxic effects of OA, competes with OA for binding to serum albumin. L-phenylalanine was found to have some protective effect for the toxic effects of OA because it diminishing OA's inhibitory effect to some of enzymes discussed earlier.

Most mycotoxins have a high affinity for hydrated sodium calcium aluminasilicate (HSCAS or NovaSil) and other related products. Diets containing NovaSil and related absorbers have been found effective in preventing absorption of AFB1 and several other mycotoxins in test animals, thus decreasing their toxicity [(see Bhatnagar et al. 2002; Huwig et al. 2001; Phillips et al. 2002)]. Likewise, several other adsorbents such as zeolite, bentonite, and superactive charcoal have been found to be effective in decreasing the toxicity of other mycotoxins such as T-2 toxin.

5 CONCLUSIONS

Mycotoxins are low molecular weight secondary metabolites of fungi that are contaminants of agricultural commodities, foods, and feeds. Fungi that produce these toxins do so both prior to harvest and during storage. Although contamination of commodities by toxigenic fungi occurs frequently in areas with a hot and humid climate (i.e., conditions favorable for fungal growth), they can also be found in temperate conditions. Production of mycotoxins is dependent upon the type of producing fungus and environmental conditions such as the substrate, water activity (moisture and relative humidity), duration of exposure to stress conditions, and microbial, insect, or other animal interactions. Although outbreaks of mycotoxicoses in humans have been documented, several of these have not been well characterized, neither has a direct correlation between the mycotoxin and resulting toxic effect been well established *in vivo*. Even though the specific modes of action of most of the toxins are not well established, acute and chronic effects in prokaryotic and eukaryotic systems, including humans have been reported. The toxicity of the mycotoxins varies considerably with the toxin, the animal species exposed to it, and the extent of exposure, age, and nutritional status. Most of the toxic effects of mycotoxins are limited to specific organs, but several mycotoxins affect many organs. Induction of cancer by some mycotoxins is a major concern as a chronic effect of these toxins. It is nearly impossible to eliminate mycotoxins from food and feed in spite of the regulatory efforts at the national

and international levels to remove the contaminated commodities. This is because mycotoxins are highly stable compounds, the producing fungi are ubiquitous, and food contamination can occur both before and after harvest. Nevertheless, good farm management practices and adequate storage facilities minimize the toxin contamination problems. A combination of natural biocontrol competition fungi and enhancement of host-resistance against fungal growth or toxin production could prevent toxin formation to a very significant extent. Rigorous programs for reducing the risk of human and animal exposure to contaminated food and feed also include economically feasible and safe detoxification processes and dietary modifications. Although risk assessment has been made for some mycotoxins (Coker 1998; DeVries et al. 2002), additional, systematic epidemiological data for human exposure is needed for establishing toxicological parameters for mycotoxins and the safe dose for humans. It is unreasonable to expect complete elimination of the mycotoxin problem. But multiple approaches will be needed to minimize the negative economic impact of the toxins on the entire agriculture industry as well as their harmful effects on human and animal health.

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Genetics and Biochemistry of Mycotoxin Synthesis

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1 INTRODUCTION

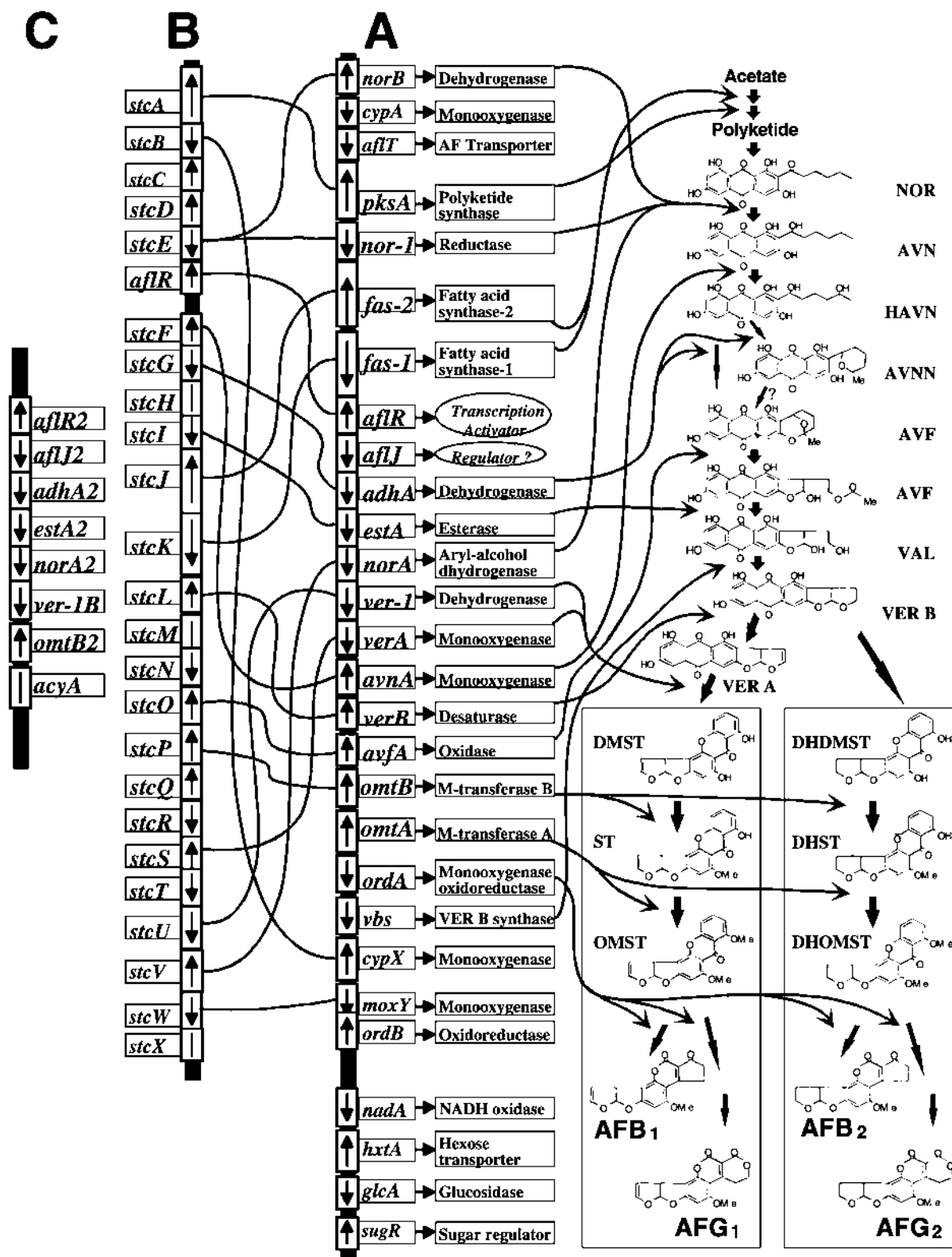
Mycotoxins are low-molecular weight, nonproteinaceous, organic secondary metabolites produced by fungi from amino acids, shikimic acid, or malonyl CoA. These compounds are toxic, mutagenic, teratogenic, and carcinogenic to animals and humans (Bennett 1987; Bhatnagar et al. 2002; Eaton and Groopman 1994; Hall and Wild 1994; Squire 1989). Over 300 mycotoxins have been identified, but only those implicated in mycotoxicoses involving humans have been studied in detail with respect to the biochemistry and genetics of their biosynthesis. Research on the natural occurrence, identification and characterization, biosynthesis and genetic regulatory control of mycotoxins, as well as prevention and control of mycotoxin contamination of food and feed gained momentum after the incidence of “Turkey-X” disease in 1960 when 10,000 turkeys died due to aflatoxin contamination in the peanut-meal feed. Due to the risk of mycotoxin contamination of foods and feed on human health and livestock productivity (Brown et al. 1998; Eaton and Groopman 1994), regulations have been established in over 50 countries for acceptable levels of mycotoxins in commodities for commerce or food and feed for human and animal consumption (Bhatnagar et al. 2002; Sharma and Salunkhe 1991). Within the last decade, significant progress toward the understanding of several mycotoxins in the world has been made. In this chapter, the genetics and biochemistry of only the most economically significant mycotoxins, aflatoxins, sterigmatocystin (ST), trichothecenes, and fumonisins, are summarized and discussed. For other mycotoxins such as alternaria toxins AAL toxins, AK-toxin, ochratoxins, citrinin, cyclopiazonic acid (CPA), patulin, paxilline, zearalenone, ergot alkaloids and related toxins, and other neurotropic mycotoxins, kindly refer to the most recent

comprehensive reviews on this subject (Bhatnagar et al. 1988; 2002).

2 AFLATOXINS AND STERIGMATOCYSTINS

2.1 Occurrence and Toxicology

Aflatoxins are toxic and carcinogenic secondary metabolites produced primarily by *Aspergillus flavus* and *A. parasiticus* as well as *A. nomius*. One isolate of each of *A. tamarii* (Goto et al. 1996) and *A. ochraceoroseus* (Frisvad and Samson 1999) have been reported to produce aflatoxins (Klich et al. 2000). The aflatoxins consist of a group of at least 16 structurally related toxins (Goldblatt 1969) characterized so far. Amongst these, aflatoxins, B₁, B₂, G₁ and G₂ (AFB₁, AFB₂, AFG₁ and AFG₂) are the major toxins. Aflatoxins are polyketide-derived, bis-furan-containing dihydrofuranofuran and tetrahydrofuran moieties (rings) fused with a substituted coumarin (Figure 1). *A. flavus* produces aflatoxins, B₁ and B₂; while *A. parasiticus* produces the four major aflatoxins, B₁, B₂, G₁ and G₂. The aflatoxins, M₁ and M₂, are modified forms of aflatoxin B₁ found in bovine milk. Aflatoxin B₁ (AFB₁) is the most potent naturally occurring toxin and carcinogen known (Squire 1989). The toxicity of the major aflatoxins has been established in the following order: B₁ > G₁ > B₂ > G₂. Aflatoxin M₁ is 10-fold less toxic than AFB₁, but its presence in milk is of concern in human health (Cullen et al. 1987; Galvano et al. 1996; Van Egmond 1989b). Due to the toxic and carcinogenic properties of aflatoxins (Busby and Wogan 1981; Eaton and Groopman 1994), these compounds are the most thoroughly studied of all the mycotoxins, and significant research has been conducted on their biosynthetic pathway



and genetic control of its regulation (Bhatnagar et al. 2002; Chang et al. 1996; Payne and Brown 1998; Yu et al. 1995a).

Sterigmatocystin and dihydrosterigmatocystin (DHST) are produced by various *Aspergilli* including *A. nidulans*; several genera *Bipolaris* and *Chaetomium*, and *Penicillium luteum* are also reported to produce ST (Cole and Cox (1987)). *Aspergillus nidulans* is an industrial fungus and has been the model organism for the study of ST biosynthesis. ST and DHST are the penultimate precursors of AFB₁, AFB₂, AFG₁ and AFG₂, respectively (Figure 1; Betina 1989; CAST 1989; Chu 1991). Like aflatoxins, ST and DHST are hepatotoxic and carcinogenic mycotoxins. However, their carcinogenicity is far less than that of aflatoxin B₁ in animal test (Mori and Kawai 1989; Van der Watt 1977). ST contaminates cereal grains (barley, rice and corn), coffee beans and cheese (Jelinek et al. 1989) and is considered a health hazard as well. Since ST and DHST are aflatoxin precursors, they share a common biochemical pathway, homologous genes and regulatory mechanism. For these reasons, aflatoxins and ST are discussed together.

2.2 The Clustering of Aflatoxin Biosynthetic Pathway Genes

Significant progress has been made in the last decade in deciphering aflatoxin biosynthetic pathway (Bennett 1981; 1987; 1986; 1994; Bhatnagar et al. 1988; 1991; 1992; 1993; 1998; Bhatnagar and Cleveland 1990; Cary et al. 2000a,b; Chang et al. 1992; 1993; 1995a,b; 2000b; Cleveland and Bhatnagar 1992; 2002; Dutton 1988; Minto and Townsend 1997; Papa 1982; 1984; Payne 1998; Payne and Brown 1998; Silva and Townsend 1996; Silva et al. 1996; Townsend 1997; Trail et al. 1995a; 1995b; Yu et al. 1993; 1995a; 1997; 1998; 2000a,b). As many as 15 structurally defined aflatoxin intermediates have been identified in the aflatoxin/ST biosynthetic pathway starting with the acetate, the polyketide precursors. At least 23 enzymatic steps involved in the aflatoxin biosynthesis have been characterized or proposed (Figure 1).

It has been long proposed that the aflatoxin pathway genes may be clustered with a common regulator (Cleveland and Bhatnagar 1991). The first experimental evidence showing the potential clustering of aflatoxin pathway genes was, however, demonstrated by Trail et al. (1995b) when they found that the *nor-1* and *ver-1* genes were linked in a cosmid clone (NorA) with the regulatory gene *aflR* and a putative aflatoxin pathway gene, *uvm8* (now named *fas-1*) in between. This observation provided evidence that at least the early stages of the pathway may be linked. By mapping overlapping cosmid clones in *A. parasiticus* and *A. flavus* (Yu et al. 1995a), a linkage of genes involved in aflatoxin formation, from early stage *nor-1* gene to later stage *omtA*, was established indicating that the entire aflatoxin biosynthetic pathway genes may be clustered. This observation and subsequent discoveries of pathway genes led to a consensus cluster map consisting of at least nine aflatoxin pathway genes *pksA*, *nor-1*, *uvm8* (now named *fas-1*), *aflR*, *ver-1*, *omtA* and three additional open reading frames (ORFs) with unknown function (*ord-1*, now named *avnA*; *ord-2*, now named *ordA*; and *aad*, now named *norA*) (Yu et al. 1995a). The concept of aflatoxin pathway gene cluster greatly accelerated the rate of gene discovery (Cary et al. 1996; Chang et al. 1995a; 2000b; Chang and Yu 2002; Feng and Leonard 1995; Mahanti et al. 1996; Silva et al. 1996; Silva and Townsend 1996; Yu et al. 1997; 1998; 2000a,b,c). It was identified that at least 23 genes (Cary et al. 1996; Chang et al. 1992; 1993; 1995a; 2000b; Chang and Yu 2002; Cleveland et al. 1997; Meyers et al. 1998; Motomura et al. 1999; Prieto and Woloshuk 1997; Silva et al. 1996; Silva and Townsend 1996; Yu et al. 1993; 1995a; 1997; 1998; 2000a,b) or ORFs involved in aflatoxin biosynthesis including the regulatory genes, *aflR* and *aflJ*, were clustered together within approximately 80 kb DNA regions in the *A. parasiticus* and *A. flavus* genomes respectively. Most of these genes on the aflatoxin pathway gene cluster have been cloned and characterized. The cloning of *cypX* and *moxY* genes (Yu et al. 2000a), the *ordB* gene (potentially for an oxidoreductase, Yu, unpublished), and the sugar utilization gene cluster (Yu et al. 2000c) defined one end of the aflatoxin pathway gene cluster (Figure 1, panel A). The *norB*, *cypA* and *aflT* might possibly mark the other end of

Figure 1 Proposed and generally accepted pathway for aflatoxin B₁, B₂, G₁ and G₂ biosynthesis and the corresponding genes and their enzymes are presented. The aflatoxin biosynthetic pathway gene cluster in *A. parasiticus* and *A. flavus* (panel A), sterigmatocystin biosynthetic pathway gene cluster in *A. nidulans* (panel B), and the non-functional, partially duplicated aflatoxin gene cluster in *A. parasiticus* (panel C) are shown. The gene names are labeled on the side of the cluster. Arrows indicate the direction of gene transcription. The homologous genes between the sterigmatocystin pathway gene cluster in *A. nidulans* and aflatoxin pathway gene cluster in *A. parasiticus* and *A. flavus* is connected by line. The four sugar utilization genes linked to the aflatoxin pathway gene cluster are on the bottom of panel A. Abbreviations for the intermediates are: norsolorinic acid (NOR), averantin (AVN), 5'-hydroxyaverantin (HAVN), averufanin (AVNN), averufin (AVF), versiconal hemiacetal acetate (VHA), versiconal (VAL), versicolorin B (VER B), versicolorin A (VER A), demethylsterigmatocystin (DMST), sterigmatocystin (ST), *O*-methylsterigmatocystin (OMST), aflatoxin B₁ (AFB₁), aflatoxin G₁ (AFG₁), DMDHST, dihydrosterigmatocystin (DHST), dihydro-*O*-methylsterigmatocystin (DHOMST), aflatoxin B₂ (AFB₂), and aflatoxin G₂ (AFG₂), Methyltransferase (M-transferase).

this cluster (Yu et al., unpublished; Chang et al., unpublished; Figure 1, panel A). Using a similar strategy, Brown et al. (1996b) defined ST biosynthetic pathway gene cluster consisting of 25 coregulated transcripts in *A. nidulans*. The ST gene cluster contains probably a complete set of ST genes within approximately 60-kb DNA fragment (Brown et al. 1996b). The aflatoxin pathway gene clusters between *A. flavus* and *A. parasiticus* are identical in term of the sequential order of the genes, the sizes of these genes and the direction of gene transcription. The nucleotide and amino acid sequences of the aflatoxin pathway genes are also highly conserved (>95%) between *A. parasiticus* and *A. flavus* (Yu et al. 1995a). Analyzing the corresponding genes and their enzymes for a specific pathway conversion step between *A. flavus*/*A. parasiticus* for aflatoxin synthesis and *A. nidulans* for ST synthesis, it has been shown a high degree of similarity with respect to the genes, the enzyme encoded by these genes and their enzyme function. However, the order of these genes on the chromosome between the two clusters are totally scrambled (Figure 1, panel A & B).

In *A. parasiticus*, duplication of aflatoxin genes *ver-1* and *aflR* was first suggested (Mehigh et al., unpublished; Liang and Linz, unpublished) and reported by Liang et al. (1996). This partial duplicated aflatoxin gene cluster consisting of seven duplicated genes, *aflR2*, *aflJ2*, *adhA2*, *estA2*, *norA2*, *ver1B*, *omtB2*, has been cloned and characterized by Chang and Yu (2002). The genes within this partial duplicated cluster, due possibly to the chromosome location (Chiou et al. 2002; Yu et al., unpublished), were found to be unfunctional under normal conditions even though no apparent defects are identified in some of these genes (*aflR2*, *aflJ2*, *adhA2*, *estA2*).

2.3 The Biosynthetic Pathways of Aflatoxins and Sterigmatocystin

2.3.1 Acetate to Norsolorinic Acid (NOR) Trail et al. (1995b)

Molecular evidence supports the plausible hypothesis that two fatty acid synthases (FAS) and a polyketide synthase (PKS) are involved in the synthesis of polyketide from acetate (Bhatnagar et al. 1992; Brown et al. 1996a; Townsend et al. 1984; Trail et al. 1995a). Complementation of an aflatoxin blocked UV mutant, UVM8, Mahanti et al. (1996) identified a gene named *uvm8*, which is required for NOR biosynthesis and aflatoxin production in *A. parasiticus*. The predicted amino acids of the gene *uvm8* shares high degree of similarity (67%) and identity (48%) to the beta-subunit of FASs (FAS1) from *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (Trail et al. 1995a,b). Complementation, metabolite feeding and disruption experiments by Mahanti et al. (1996) confirmed that the 7.5-kb large transcript of the gene *uvm8* encodes one subunit of a novel FAS directly involved in the backbone formation prior to *nor-1* of aflatoxin synthesis. For the proposed function, the gene *uvm8* was renamed *fas-1A*. The *fas-1A* gene was renamed *fas-1* encoding FAS-1 in the

aflatoxin biosynthetic pathway gene cluster (Figure 1, panel A). Sequence analyses have shown that there are two large genes, *fas-1A* and *fas-2A*, encoding for beta- and alpha-subunit of FAS, respectively (Mahanti et al. 1996, unpublished, personal communication). The gene *fas-1A* and *fas-2A* were renamed *fas-1* and *fas-2* in the aflatoxin pathway gene cluster encoding for FAS-1 (FAS α) and FAS-2 (FAS β), respectively (Payne and Brown 1998). Brown et al. (1996a) proposed the involvement of FAS in ST biosynthesis in *A. nidulans*. They identified two genes, *stcJ* and *stcK* in the ST cluster, that are homologous to FASs, *fas-2* and *fas-1* of aflatoxin pathway genes, respectively. Disruption of *stcJ* and *stcK* encoding FAS α and FAS β subunits (FAS-2 and FAS-1), respectively in *A. nidulans*, stopped ST synthesis. Watanabe et al. (1996) provided the biochemical evidence for the role of a FASs and PKSs in the biosynthesis of aflatoxin. The N-acetylcysteamine thioester of hexanoic acid could be incorporated into NOR in a disrupted *fas-1* transformant. Mayorga and Timberlake (1992) identified a gene *wA* encoding the PKS in *A. nidulans*. Chang et al. (1995a) confirmed that a PKS is required for aflatoxin biosynthesis by cloning the *pksA* gene encoding a PKS for the synthesis of polyketide from *A. parasiticus*. Feng and Leonard (1995) also isolated a *pksL1* gene for PKS, the equivalent of *pksA* gene, by PCR from a pool of 19 *A. parasiticus* clones. Disruption of the *pksL1* gene produced no aflatoxin or any aflatoxin intermediates. The *pksL1* gene is 99% identical to *pksA* and they are believed to be the same gene. The 1% sequence discrepancy may possibly be due to strain variation and sequencing errors. Yu (1995) isolated a gene, *pksST* that encodes a PKS from *A. nidulans*. The nucleotide sequence of *pksST* from *A. nidulans* is identical to *stcA* (Brown et al. 1996b). However, no significant sequence homology was found between *wA* and *stcA* (*pksST*). The predicted amino acid sequences of these PKS contains a typical four conserved domains common to other known PKS proteins: β -ketoacyl synthase (KS), acyltransferase (AT), acyl carrier protein (ACP), and thioesterase (TE). The gene for PKS from *A. parasiticus* (*pksA* or *pksL1*) was designated as *pksA* (Yu et al. 1995a) in the aflatoxin pathway gene cluster and its homolog in *A. nidulans* was designated as *stcA* (Brown et al. 1996b). NOR is the first stable intermediate in the pathway (Bennett 1981; Bennett et al. 1997; Papa 1979; 1982). However, the predicted conversion product of PKS in the aflatoxin pathway is believed to be a noranthrone. The conversion for noranthrone to NOR is poorly defined, but it has been proposed to occur by a noranthrone oxidase (Vederas and Nakashima 1980), a monooxygenase (Bhatnagar et al. 1992), or spontaneously (Dutton 1988).

2.3.2 NOR to Averantin (AVN)

Using NOR-accumulating mutants, it was demonstrated by Papa (1979); Papa (1982) in *A. flavus* and by Bennett (1981), Detroy et al. (1973) in *A. parasiticus* that NOR was an intermediate in aflatoxin biosynthetic pathway (Bennett et al. 1997). It was found that the NOR-accumulating mutants were

always leaky without completely blocking aflatoxin biosynthesis. Bhatnagar et al. (1992) proposed the involvement of dehydrogenase in the conversion of NOR to AVN and Chang et al. (1992) identified and cloned the gene, *nor-1*, that complemented a NOR-non-producing mutant in *A. parasiticus*. Trail et al. (1994) further characterized this gene to encode an enzyme that functions as a ketoreductase for the conversion of NOR to AVN. Cary et al. (1996) characterized a gene, *norA* encoding an aryl-alcohol dehydrogenase in the aflatoxin pathway gene cluster, which were involved in the conversion of NOR to AVN (Bennett et al. 1980; Bennett 1981; Bhatnagar et al. 1992). An additional gene, *norB*, which was also found to be homologous to the *nor-1* and *norA* genes in the aflatoxin pathway gene cluster in *A. parasiticus* (Yu et al. unpublished). The enzymatic function, and coordinated genetic regulation of the three NOR-converting genes are to be further investigated. The *nor-1* homologous gene in *A. nidulans* is *stcE* (Brown et al. 1996b).

2.3.3 AVN to 5'-hydroxyaverantin (HAVN)

Earliest evidence for the conversion of AVN to HAVN was elucidated via radiotracing by Bennett et al. (1980), McCormick et al. (1987). Subsequently, Yabe et al. (1991b), based on the enzymatic studies, proposed three enzymatic steps in two possible routes involved in the conversion of aflatoxin intermediates from NOR to AVF: (a) conversion from NOR to AVN catalyzed by a dehydrogenase; (b) conversion from NOR to HAVN by a monooxygenase; (c) HAVN then would be converted to AVF by a second dehydrogenase. Yabe et al. (1991b) also proposed that this reaction was reversible and that the NADPH was the preferred cofactor. Bhatnagar et al. (1992) proposed two alternate routes requiring 3–4 enzymes for the conversion of NOR to AVF. Yu et al. (1997) cloned and disrupted a gene that encodes a P-450 monooxygenase (previously named *ord-1*, Yu et al. 1995a). Using disruption and substrate feeding it was defined that HAVN and averufanin (AVNN) are the conversion intermediate products from AVN to averufin (AVF). This gene was designated *avnA*, which has high sequence similarity to the *stcF* gene in the *A. nidulans* ST cluster (Brown et al. 1996b).

2.3.4 HAVN to AVNN and AVF

In the proposed scheme by Yabe et al. (1991b; 1993), AVNN was considered a shunt metabolite and not an aflatoxin intermediate. Bhatnagar et al. (1992) proposed both AVN and AVNN were the intermediates from NOR to AVF based on radiolabeling experiment. Chang et al. (2000b) cloned and characterized a gene, *adhA*, encoding an alcohol dehydrogenase in *A. parasiticus*. Gene disruption and feeding experiments demonstrated that AVN might be converted directly to AVF or indirectly to AVF through an intermediate substrate AVNN. The exact nature of the enzymatic function and the possible involvement of additional enzymes are still

unclear. Woloshuk and Payne (1994) identified an alcohol dehydrogenase gene, *adh1*, in *A. flavus* that is expressed concurrently with aflatoxin pathway genes. The *adh1* gene in *A. flavus* and *adhA* gene in *A. parasiticus* shares no significant homology at both DNA and amino acid level. The involvement of the *adh1* from *A. flavus* in aflatoxin synthesis is to be investigated.

2.3.5 AVF to Versiconal Hemiacetal Acetate (VHA)

The conversion from AVF to VHA is thought to involve an oxidase (Bhatnagar et al. 1992). Yu et al. (2000b) cloned the *avfA* gene from both *A. parasiticus* and an AVF-accumulating *A. flavus* strain as well as *A. sojae* strain that was shown by gene complementation experiment to encode for an enzyme (homologous to oxidase) responsible for the conversion from AVF to VHA. The *avfA* gene homolog in *A. nidulans* was identified to be *stcO* (Yu et al. 2000b).

2.3.6 VHA to Versiconal (VAL)

The evidence for the involvement of an esterase in the conversion of VHA to VAL in aflatoxin biosynthesis was demonstrated by Bennett et al. (1976), Fitzell et al. (1977), Schroeder et al. (1974), Yabe et al. (1991a,b), and Yao and Hsieh (1974), when the *A. parasiticus* was treated with the organophosphorus pesticide dichlorvos. The esterase has been purified in *A. parasiticus* by Hsieh et al. (1989); Kusumoto and Hsieh (1996). Yu et al. (2003) cloned a gene, *estA*, which could well be the gene for this esterase. The homologous gene in *A. nidulans* ST biosynthetic gene cluster is *stcI*. Attempts to disrupt the *estA* or *stcI* have not resulted in any conclusive evidence for the involvement of these genes in toxin synthesis.

2.3.7 VAL to Versicolorin B (VER B)

Yabe and Hamasaki (1993) provided enzymatic evidence for the conversion of VAL to VER B. Silva et al. (1996), Silva and Townsend (1996), and McGuire et al. (1996) cloned, characterized and expressed the *vbs* gene in the aflatoxin pathway gene cluster in *A. parasiticus*. It was demonstrated that the VER B synthase catalyzes the side chain cyclodehydration of racemic VHA to VER B. This is a key step in the aflatoxin formation since it closed the bisfuran ring of aflatoxin for binding to DNA. The homologous gene in *A. nidulans* ST biosynthetic gene cluster is *stcN*.

2.3.8 VER B to Versicolorin A (VER A)

In the aflatoxin biosynthetic pathway, the VER B is a critical branch point (Bhatnagar et al. 1991) leading either to AFB₁ and AFG₁ or to AFB₂ and AFG₂. The conversion of VER B to VER A was proposed to require a desaturation of the bisfuran ring of VER B (Bhatnagar et al. 1993; Yabe and Hamasaki 1993). Disruption of *stcL* in *A. nidulans* by Kelkar et al.

(1997) stopped ST synthesis but resulted in the accumulation of VER B. The *stcL* gene, for a P-450 monooxygenase, was shown to be required for the conversion of VER B to VER A. A homologous gene to *A. nidulans stcL* in the aflatoxin pathway gene cluster, *verB*, encoding a P-450 monooxygenase/desaturase, was cloned from *A. parasiticus* and *A. flavus* strains (Bhatnagar et al., unpublished, GenBank accession numbers: AF106958, AF106959, and AF106960 respectively).

2.3.9 VER a to Demethylsterigmatocystin (DMST) and VER B to Demethyldihydrosterigmatocystin (DMDHST)

Another cloned gene involved in a key step aflatoxin synthesis is the *ver-1* gene (Skory et al. 1992) in *A. parasiticus*, this gene was shown by complementing the *ver-1* mutant to be required for the conversion of VER A to DMST in *A. parasiticus*. Keller et al. (1994) identified a gene *stcU* (formerly named *verA*) in *A. nidulans*, which is a homolog of *ver-1* in *A. parasiticus* for a ketoreductase, required for the conversion of VER A to DMST. Double mutation of *stcU* and *stcL* resulted accumulation of only VER B. Keller et al. (1995a) also identified *stcS* (formerly named *verB*, Keller et al. 1995b), which is homologous to P-450 monooxygenases, to be involved in the conversion of VER A to DMST. Disruption of this gene resulted in the accumulation of VER A. Thus, both *stcU* and *stcS* are required for the conversion of VER B to DMST. The *stcS* homolog, named *verA* in *A. parasiticus* strain, was also identified (Yu, unpublished).

2.3.10 DMST to ST and DMDHST to DHST

Yabe et al. (1989) demonstrated a distinct methyltransferase activity in *A. parasiticus* for the conversion of DMST to ST. This enzyme may also be responsible for the conversion of DMDHST to DHST. The enzyme was purified and characterized (Yabe et al. 1998; Yabe et al. 1999). Disruption of *stcP* in *A. nidulans* by Kelkar et al. (1996) showed the requirement of this gene for the conversion from DMST to ST. The gene responsible for the conversion of DMST to ST and DMDHST to DHST were cloned by Motomura et al. (1999), named *dmtA* for *O*-methyltransferase I in *A. parasiticus* and concurrently by Yu et al. (2000b), named *omtB* for *O*-methyltransferase B in *A. parasiticus*, *A. flavus* and *A. sojae*.

2.3.11 ST To *O*-methylsterigmatocystin (OMST) and DMST to Dihydro-*O*-methylsterigmatocystin (DHOMST)

Several researchers (Bhatnagar et al. 1988; Keller et al. 1993; Yabe et al. 1989) reported the presence of an *O*-methyltransferase for the conversion of ST to OMST and DHST to DHOMST. Yu et al. (1993) cloned the cDNA

sequence from *A. parasiticus* (initially named *omt-1*, later renamed as *omtA*) by reverse genetics. The enzyme was expressed in *Escherichia coli* and its activity to convert ST to OMST was demonstrated by substrate feeding studies. The genomic DNA sequence of this gene (*omtA*) was cloned from *A. parasiticus* and *A. flavus* (Yu et al. 1995b). This *omtA* gene homologue was also detected in other aflatoxigenic and nonaflatoxigenic *Aspergillus* species (Klich et al. 1995).

2.3.12 OMST to Aflatoxin B₁ (AFB₁) and Aflatoxin G₁ (AFG₁) and DMDHST to Aflatoxin B₂ (AFB₂) and Aflatoxin G₂ (AFG₂)

By feeding experiments, Yabe et al. (1988a) proposed the relationship between B-Group (AFB₁ and AFB₂) and G-Group (AFG₁ and AFG₂) aflatoxin biosynthesis. It was also predicted by Bhatnagar et al. (1992) that in the late stages of aflatoxin biosynthesis, a NADPH-dependent monooxygenase is required for the conversion of OMST to AFB₁. Prieto et al. (1996), Prieto and Woloshuk (1997) reported in *A. flavus* that a P-450 monooxygenase gene, *ord-1*, is required for this reaction. Yu et al. (1998) cloned the P-450 monooxygenase gene, *ordA*, from *A. parasiticus* and an *A. flavus* mutant strain and demonstrated by expression and substrate feeding in yeast system that this gene is responsible for the conversion of OMST to AFB₁ and AFG₁, and DHOMST to AFB₂ and AFG₂. In this study, the critical amino acids for the enzymatic activity and heme-binding motif were identified by site-directed mutagenesis as well. Yu et al. (1998) also demonstrated that additional enzyme(s) is required for the G-group (AFG₁ and AFG₂) aflatoxin synthesis. However, the enzyme(s) and corresponding gene(s) have not been isolated as yet. It should be noted that the functions of three additional genes in the aflatoxin pathway gene cluster, *cypA* (Yu, unpublished), *cypX* (*cypX* = *stcB*) and *moxY* (*moxY* = *stcW*) encoding cytochrome P-450 monooxygenases and monooxygenase (Yu et al. 2000a), have not yet been assigned. Keller et al. (2000) examined several characterized cytochrome P-450 monooxygenases and proposed the functions for some of the identified genes or ORFs encoding monooxygenases in aflatoxin/ST synthesis. However, there is a possibility that these genes might be involved in the G-group toxin formation.

2.4 Factors Affecting Aflatoxin/ST Biosynthesis

2.4.1 Genetic Regulation

Since the aflatoxin and ST biosynthetic pathway genes are found to be clustered on a single chromosome in both *A. parasiticus* and *A. flavus* and in *A. nidulans*, respectively, (Brown et al. 1996b; Woloshuk and Prieto 1998; Yu et al. 1995a), these genes are presumably expressed concurrently in the genome. In both the aflatoxin and ST gene clusters, there is a positive regulatory gene, *aflR*, for activating pathway gene transcription located in the middle of the gene clusters.

The *aflR* gene, coding for a sequence specific zinc binuclear DNA-binding protein, a Gal 4-type 47 kDa polypeptide, has been shown to be required for transcriptional activation of most, if not all, the structural genes (Chang et al. 1993; 1995b; 1999a,b; Ehrlich et al. 1998; Flaherty and Payne 1997; Payne et al. 1993; Yu et al. 1997; Woloshuk et al. 1994) by binding to the palindromic sequence 5'-TCGN5CGA-3' in the promoter region of the structural genes (Ehrlich et al. 1999a,b; Fernandes et al. 1998) in *A. parasiticus*, *A. flavus*, and *A. nidulans* (Yu et al. 1996). In *A. sojae*, a nontoxigenic strain used in industrial fermentations, was found to contain a defective *aflR* gene in addition to potential other defects (Matsushima et al. 2001a,b; Takahashi et al. 2002). Thus, with the absence of the functional regulatory protein, no induction of aflatoxin can occur in this food grade *Aspergillus*. Additional factors involved in regulation of aflatoxin synthesis were evidenced by Flaherty and Payne (1997). Adjacent to the *aflR* gene in the aflatoxin gene cluster, a divergently transcribed gene, *aflJ*, was also found to be involved in the regulation of transcription (Meyers et al. 1998; Chang, personal communication). The exact mechanism by which *aflJ* modulates transcription of these pathway genes in concert with *aflR* is to be studied. A gene, *aflT*, encoding a membrane bound protein with homology to antibiotic efflux genes presumed to be for transporting aflatoxin out of the fungal cell, was discovered in *A. parasiticus* (Chang and Yu, unpublished, GenBank accession number: AF268071). The *aflT* gene might be involved in someway in aflatoxin secretion.

2.4.2 Nutritional Control (Carbon and Nitrogen Sources)

The best-known nutritional factors affecting aflatoxin biosynthesis are carbon and nitrogen sources (Adye and Mateles 1964; Bennett et al. 1979; Luchese and Harrigan 1993). It is clear that simple sugars such as glucose, sucrose, fructose, and maltose support aflatoxin formation, while peptone and lactose are not (Buchanan and Stahl 1984; Payne and Brown 1998). Woloshuk et al. (1997) reported the connection between alpha amylase activity and aflatoxin production in *A. flavus*. Yu et al. (2000c) identified a group of four genes that constitute a well-defined gene cluster related to sugar utilization in *A. parasiticus* next to the aflatoxin pathway gene cluster. The expression of the *hxtA* gene, encoding a hexose transporter protein, was found to be concurrent with the aflatoxin pathway cluster genes in aflatoxin-conductive medium. This is the first evidence that primary metabolism (sugar metabolism) and secondary metabolism (aflatoxin biosynthesis) are genetically linked on the chromosome. A close physical linkage between the two gene clusters could point to a relationship between the two clusters in reference to the processing of carbohydrates leading to the induction of aflatoxin biosynthesis. Lipid substrate was shown to be a good carbon source to support aflatoxin production (Fanelli and Fabbri 1989; Fanelli et al. 1983; 1995). A lipase gene, *lipA*, was cloned by Yu et al. (unpublished) in *A. parasiticus* and *A. flavus*. It was demonstrated that this

lipase gene expression and subsequent aflatoxin production are induced by lipid substrate. Non-aflatoxin-conductive peptone medium supplemented with 0.5% soybean oil induces lipase gene expression and leads to aflatoxin formation (Yu et al. unpublished).

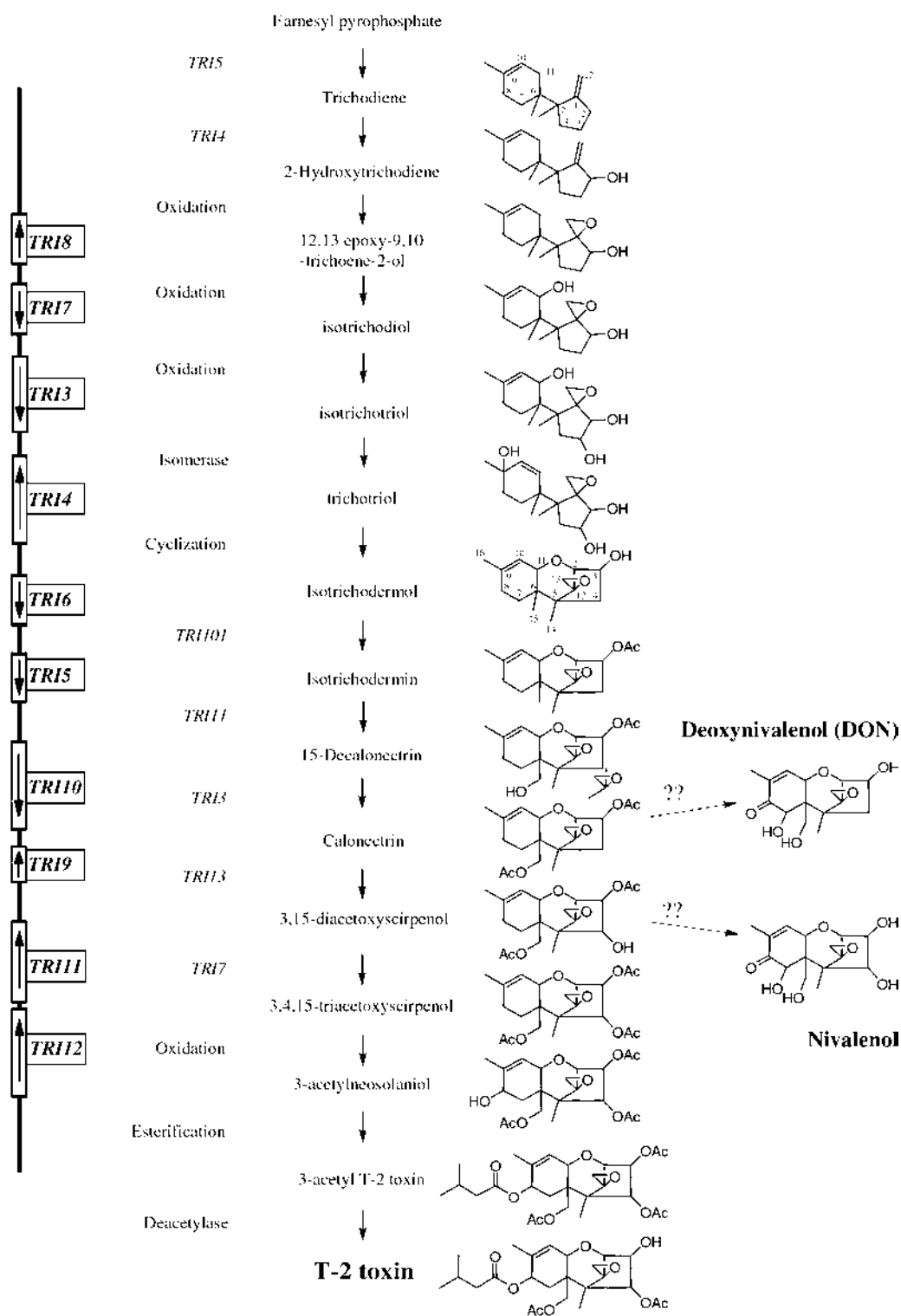
Nitrogen source plays an important role in aflatoxin production. Asparagine, aspartate, alanine, ammonium nitrate, ammonium nitrite, ammonium sulfate, glutamate, glutamine, and proline containing media support aflatoxin production; while sodium nitrate and sodium nitrite containing media do not (Davis et al. 1967; Eldridge 1964; Payne et al. 1983; Reddy et al. 1971; 1979). Studies by Kachholz and Demain (1983), Niehaus and Jiang (1989) suggested that nitrate represses AVF and aflatoxin formation. It was shown that while high temperature and nitrate support ST production in *A. nidulans*, the opposite was seen with *A. parasiticus* where these culture conditions repress synthesis of aflatoxin (Feng and Leonard 1998). Studies by Chang et al. (1995b) demonstrated that nitrate has suppressive effect on aflatoxin production and overexpression of *aflR* gene by additional copies of *aflR* could be required to overcome the negative regulatory effect on aflatoxin pathway gene transcription in the nitrogen control circuit. Chang et al. (1996) cloned a nitrogen utilization gene cluster consisting of two genes, *niaD* and *niiA* from *A. parasiticus*, *A. oryzae*, *A. niger* and *A. nidulans* and a nitrogen regulator, *areA* (Chang et al. 2000a) from *A. parasiticus*. In the intergenic region between *aflR* and *aflJ* several AreA binding motifs have been identified (Chang et al. 2000a). The AreA binding could prevent AflR binding. It seems that the aflatoxin formation and nitrogen metabolism are closely linked.

2.4.3 Environmental Influences

Aflatoxin production are also affected by many environmental factors (Bennett and Papa 1988; Demain 1972; Kale et al. 1994; Kale et al. 1996; Yabe et al. 1988b) such as physiological pH, temperature and water activity in the environment, volatile metabolites produced in host plant in response to fungal invasion, and developmental stages of the fungi.

Fungi have the ability to maintain internal pH and respond to the environmental changes. Studies by Cotty (1988) demonstrated the pH effect on aflatoxin formation and sclerotia development. Acidic pH condition favors aflatoxin biosynthesis in response to pH (Espeso et al. 1993; Keller et al. 1997). It is believed that *pacC* is a major transcriptional regulatory factor (Keller et al. 1997). In the promoter region of the regulatory gene, *aflR*, at least one *pacC* binding site has been identified (Ehrlich et al. 1999a). In the non-aflatoxin-inducing peptone medium, it was shown to be inhibitory to aflatoxin formation (Cotty 1988). The regulatory mechanism might be due to the binding of *pacC* to the PACC site in the *aflR* promoter region to repress the transcription of acid-expressed gene *aflR* and the aflatoxin formation (Espeso et al. 1993) since such medium keeps the medium (pH 7.4) at alkaline condition (Cotty 1988).

Cluster Gene/Activity Intermediate Metabolite



High water activity favors spore germination and mycelial growth. However, severe aflatoxin outbreak in corn was documented under hot weather and drought conditions (Brown et al. 1998). The mechanism of *A. flavus* infestation in corn under these conditions is not well understood. The possible scenarios may include a combination of these factors: (a) the plant defense mechanism is weakened under water stress conditions; (b) higher insect feeding and associated injuries to plant tissues, thus providing entry opportunities for fungal invasion; and (c) more fungal spores dispersed in the air under drier climate conditions.

Plant metabolites also play some role on aflatoxin formation. Zeringue (1991; 1996; 1993) reported plant metabolism and aflatoxin production. Wright et al. (2000) reported that at certain conditions, *n*-Decyl aldehyde reduces not only fungal growth of *A. parasiticus* but also aflatoxin production by over 95% compared with control. Octanal reduces fungal growth by 60%, however, increases aflatoxin production by 500%, while hexanal reduces fungal growth by 50%, but shows no effect on aflatoxin production. A relationship between fungal development (production of reproductive spore and survival structure sclerotia) and aflatoxin synthesis was studied (Garber and Cotty 1997). Wilson et al. (2001) reported that the 13(*S*)-hydroperoxide derivative of linoleic acid, the reaction product of lipoxygenase (encoded by L2 LOX gene from maize), involved in reducing aflatoxin production. The fungal morphology was significantly altered and toxin synthesis was inhibited during strain degeneration studies. The effect included the inhibition of expression of the toxin pathway genes, including *aflR*.

3 TRICOTHECENES

3.1 Occurrence and Toxicology

The trichothecenes constitute a class of over 80 different members of sesquiterpenoid compounds produced by a number of genera of filamentous fungi. Based on the presence of a macrocyclic ester or ester-ether bridge between C-4 and C-15, trichothecenes are generally classified as macrocyclic (Type C) or non-macrocyclic (Types A and B). The trichothecenes, including diacetoxyscirpenol (DAS), deoxynivalenol (DON), and T-2 toxin, are mainly produced by *Fusarium* species (Bhatnagar et al. 2002; Brown et al. 2001; Chu 1997; Proctor 2000; Sharma and Kim 1991). The more structurally complex macrocyclic trichothecenes are produced by the fungal genera *Myrothecium*, *Stachybotrys*, and *Trichothecium*. *Trichoderma*, *Cephalosporium*, and

Verticimonosporium are also found to produce the trichothecenes. The most potent trichothecenes are contact toxins and cause severe blistering and necrosis of the target tissue. Their cytotoxicity parallels their acute toxicity in animals with T-2 toxin being more potent than nivalenol. Nivalenol is much more potent as an acute toxin than is DON. These trichothecenes are of great concern for food and feed contamination. DON is of considerable importance to agricultural economies because animals refuse to eat contaminated grain. *F. sporotrichioides* and *F. graminearum* are the best studied among the trichothecene producing fungi which are responsible for trichothecene contamination of grains such as maize, wheat, barley, and rye (Brown et al. 2001; Chu 1997; Proctor 2000).

3.2 Clustering of Trichothecene Biosynthetic Pathway Genes

The trichothecene biosynthesis involves multiple enzymatic reactions controlled by multiple genes. Biochemical and genetic studies of trichothecene pathway, initially in *F. sporotrichioides* using overlapping cosmid clones has resulted in establishing the consensus map of the trichothecene pathway gene cluster on a 25 kb region of the *F. sporotrichioides* chromosome (Brown et al. 2001; Hohn et al. 1993a; Proctor 2000; Trapp et al. 1998). This gene cluster consists of ten genes with the designation, in sequential order, *TRI8*, *TRI7*, *TRI3*, *TRI4*, *TRI6*, *TRI5*, *TRI10*, *TRI9*, *TRI11*, and *TRI12*, respectively (Figure 2, Brown et al. 2001). In *F. graminearum*, a similar gene cluster has been identified except that *TRI7* in *F. graminearum* is nonfunctional (Brown et al. 2001; Matsumoto et al. 1999; Proctor et al. 1995a; 1997). The gene organization and direction of transcription are identical in both *F. sporotrichioides* and *F. graminearum*. In both *F. sporotrichioides* and *F. graminearum*, the eleventh gene involved in trichothecene biosynthesis, *TRI101*, was isolated outside the established trichothecene pathway gene cluster by Kimura et al. (1998a), McCormick et al. (1999). Wuchiyama et al. (2000) identified the *TRI12* counterpart gene in *F. graminearum* designated, *TRI102*, which encodes a trichothecene efflux pump. Lee et al. (2001) identified a similar trichothecene gene cluster in *Gibberella zeae* in the order of, *TRI8*, *TRI7*, *TRI3*, *TRI4*, *TRI6*, *TRI5*, *TRI10*, *TRI9*, *TRI11*, and *TRI102*, respectively. The *TRI102* in *Gibberella zeae* shares significant homology with the *TRI12* and *TRI102* in *F. sporotrichioides* and *F. graminearum*. However, the *TRI102* in *Gibberella zeae* was found to be nonfunctional

Figure 2 Biochemical pathway and the specific genes and their enzymes involved in trichothecene biosynthesis in *Fusarium* species. Note that arrows in the gene cluster indicate the direction of gene transcription. (Figure courtesy Daren W. Brown, with modifications from Brown et al. 2001).

(Lee et al. 2001). Note that *TRI1* and *TRI2* were named based on UV-generated nontrichothecene-producing mutants. So far no corresponding gene or DNA sequences on molecular level have been identified yet and the location of these mutated loci are still unclear (Robert H. Proctor, personal communication). The functions of most of the identified genes have been proposed or characterized except *TRI9* which encodes the smallest peptide consisting of only 43 amino acids with its function unknown (Figure 2, Brown et al. 2001).

3.3 Biosynthetic Pathways of Trichothecenes

From the first trichothecene skeleton, trichodiene, to the final product, T-2 toxin, through a series chemical modifications of oxidation, isomerization, cyclization, and esterification reactions, at least 16 known stable intermediates, which constitute the various members of the trichothecenes including the branch products DON and nivalenol, have been identified. The biochemical conversion scheme of these compounds are trichodiene, 2-hydroxytrichodiene, 12,13 epoxy-9,10-trichodiene-2-ol, isotrichodiol, trichotriol, isotrichodermol, isotrichodermin, 15-decalonecetrin, calonectrin, 3,15-DAS, 3,4,15-triacetoxyscirpenol, 3-acetoxyscirpenol, 3-acetyl T-2 toxin, T-2 toxin. The DON is thought to have branched from 15-decalonecetrin and the nivalenol is thought to have branched from 3,15-DAS (Brown et al. 2001; Proctor 2000). DON could be converted directly to nivalenol (Figure 2).

The initial step of the trichothecene biosynthesis involves the incorporation of three molecules of mevalonate into the trichothecene nucleus of trichothecin with the cyclization of farnesyl pyrophosphate to produce trichodiene. *TRI5* is the first characterized pathway gene that encodes a trichodiene synthase involved in this initial step of trichothecene biosynthesis (Hohn and Beremand 1989). Hohn and Desjardins (1992) isolated a gene, named *Tox5*, encoding a trichodiene synthase for the synthesis of trichodiene from farnesyl pyrophosphate. The *Tox5* gene (renamed *TRI5*) was characterized in great detail by Desjardins et al. (1987), Desjardins et al. (1993), Cane et al. (1995), and Hohn et al. (1993b). The predicted amino acid sequences of the *TRI4* and *TRI11* gene products were similar to those of cytochrome P-450 monooxygenases (Alexander et al. 1998; Hohn et al. 1995). *TRI4* was found to catalyze the oxygenation of trichodiene at the C-2 position to yield 2-hydroxytrichodiene (Hohn et al. 1995). The most significant oxidation step in trichothecene biosynthesis is provided by this cytochrome P-450 monooxygenase encoded by *TRI4* to introduce the C-12, 13 epoxide. Alexander et al. (1998) demonstrated that the *TRI11* is involved in the conversion of isotrichodermin to 15-decalonecetrin by oxygenating the C-15 position of isotrichodermin. Studies by McCormick et al. (1999) indicated that the acetylation of isotrichodermin is catalyzed by *TRI101*. This gene may also play a role in self-protection against the toxic effect of trichothecene to the fungus by modulating trichothecene production (Muhitch et al. 2000).

By gene disruption experiment, the *TRI3* gene was found to encode an acetyltransferase catalyzing the conversion of 15-decalonecetrin to calonectrin via acetylation at the C-15 position (McCormick et al. 1996). Gene disruption and precursor feeding studies in *F. sporotrichioides* by Brown et al. (2001) demonstrated that *TRI7* might function as C-4 acetylase or acylesterase involved in the conversion from 3,15-DAS to 3,4,15-triacetoxyscirpenol. However, no functional enzyme encoded by *TRI7* was predicted in *F. graminearum* due to the lack of initiation codon, six possible frameshifts, and early stop codon (Brown et al. 2001). Brown et al. (2001) demonstrated that disruption of the *TRI8* gene failed to produce T-2 toxin instead accumulated 4,15-DAS. The chemical reaction was presumably involved in the 3-deacetylation of 3,4,15-DAS. Since the predicted amino acid sequence is homologous to lipase, the *TRI8* might encode an esterase/oxygenase and be responsible for esterification of the C-8 oxygen for the conversion somewhere between 3,4,15-triacetoxyscirpenol and 3-acetyl T-2 toxin. *TRI12* encode a toxin efflux pump (Alexander et al. 1999). *TRI6* is a regulatory gene (Desjardins et al. 1993; Hohn et al. 1999; Keller and Hohn 1997; Matsumoto et al. 1999; Proctor et al. 1995b; Proctor 2000; Trapp et al. 1998). *TRI10* encodes a polypeptide of 420 amino acids for another regulatory protein (Tag et al. 2001). The regulatory function of the *TRI6* and *TRI10* genes will be discussed in more detail in the next section. The enzymes responsible for some of the biosynthetic steps, such as the last step for the synthesis of T-2 toxin from 3-acetyl T-2 toxin by a deacetylase, have been proposed. However, the genes for those enzymes have not yet been identified (Brown et al. 2001). Most recently, two additional new genes, *TRI13* (from *Gibberella zeae*) and *TRI14* (from *F. sporotrichioides*), involved in trichothecene biosynthesis, have been isolated and deposited in the GenBank database (Daren W. Brown et al., personal communication).

Synthesis of the structurally more complex macrocyclic trichothecenes such as roridin E, verrucrin, and baccharinoid B7 is most commonly by the genus *Myrothecium*. The macrocyclic trichothecenes exhibit about 10-fold more toxic than the *Fusarium* trichothecenes. Trapp et al. (1998) identified, in *M. roridum*, the homologues of the *F. sporotrichioides* *TRI5* (*MrTRI5*), *TRI4* (*MrTRI4*), and *TRI6* (*MrTRI6*) genes which were located within a region of about 40 kb in *M. roridum* compared to only 8-kb in the *F. sporotrichioides*. The deduced amino acid sequence of the *MrTRI5* product demonstrated a homology of 72% to 75% to trichodiene synthases from four *Fusarium* species. The predicted *MrTRI6* gene product was twice as large as that of the *FsTRI6* with low amino acid homologies except in the C-terminal region of *MrTRI6* (78%) where the zinc-finger motifs reside.

3.4 Regulation of Trichothecene Biosynthesis

The *TRI6* gene is located in the middle of the trichothecene gene cluster in both *F. sporotrichioides* and *F. graminearum* (Proctor et al. 1995b). The *TRI6* gene encodes a pathway-

specific regulatory protein required for the expression of trichothecene biosynthetic pathway genes (Desjardins et al. 1993; Hohn et al. 1999; Keller and Hohn 1997; Matsumoto et al. 1999; Proctor et al. 1995b; 2000; Trapp et al. 1998). The predicted polypeptides encoded by *TRI6* in both *F. sporotrichioides* and *F. graminearum* consist of 217 and 218 amino acids, respectively. In the C-terminus of this protein, the Cys₂His₂ type zinc-finger motifs, which define the function of this particular enzyme as a gene expression regulator, were identified. *TRI6* was shown to bind to the promoter regions of nine pathway genes at the minimum consensus site 5'YNAGGCC-3' (Hohn et al. 1999). *TRI6* as a regulatory gene for binding of the protein to promoter region was supported by the study using site-directed mutagenesis in the predicted zinc-finger motif of the C-terminus of *TRI6*. Studies by Tag et al. (2001) showed that *TRI10* is another novel regulatory gene that regulates trichothecene gene expression and trichothecene synthesis. *TRI10* gene encodes a protein consisting of 420 amino acids, which does not match significantly to any proteins of known or predicted function or to motifs except a single transmembrane domain (Tag et al. 2001). Disruption of *TRI10* in *F. sporotrichioides* significantly decreased the transcript accumulation of the five tested genes, *TRI4*, *TRI5*, *TRI6*, *TRI101*, and the farnesyl pyrophosphate synthetase (*Fpps*) gene, and abolished T-2 toxin production. Complementation of *TRI10* into the disrupted mutant elevated the transcript accumulation of the above five genes and significantly increased T-2 toxin production (Tag et al. 2001). Interestingly, disruption of *TRI6* decreased transcription of other trichothecene pathway genes but increased *TRI10* transcription. *TRI10* is required for the full expression of trichothecene pathway genes including the regulatory gene *TRI6* and the expression of primary metabolic pathway genes (*Fpps*) that precedes the trichothecene biosynthetic pathway. *TRI6* is not required for *TRI10* expression but inhibits *TRI10* expression. *TRI10* may also be involved in self protection of trichothecene toxin (Tag et al. 2001). *TRI12* encodes a trichothecene efflux pump with sequence similarities to known members of the major toxin transporters. Characterization of the *TRI12* gene by Alexander et al. (1999) showed that disruption of *TRI12* resulted in reduced growth on complex media and reduced levels of trichothecene biosynthesis. Expression of *TRI12* and *TRI3* in yeast mutant (defective in the PDR5 transporter which is believed to be for toxin resistance in yeast) indicated that the role of *TRI12* in *F. sporotrichioides* functions as self-protection against trichothecenes compared with the expression of *TRI3* alone in this yeast mutant. An additional gene, *TRI101* from *F. graminearum*, which is located outside the established trichothecene gene cluster, appears to play a role in self-protection against trichothecenes (Kimura et al. 1998b; McCormick et al. 1999). Disruption of *TRI101* in *F. sporotrichioides* (*FsTRI101*) leads to reduced-growth on trichothecene-containing media compared to the wild-type strain suggesting a similar role for *FsTRI101* in

self-protection against trichothecenes (Kimura et al. 1998b; McCormick et al. 1999).

4 FUMONISINS

4.1 Occurrence and Toxicology

Investigation of the association of human esophageal cancer and the consumption of *Fusarium verticillioides* (formerly called *F. moniliforme*) infested maize by South African Researchers suggested that fumonisins were a new group of mycotoxins (Bezuidenhout et al. 1988; Gelderblom et al. 1988). Even though the fumonisins were originally identified from *F. verticillioides*, these toxins have been reported in cultures of *F. anthophilum*, *F. dlamini*, *F. napiforme*, *F. oxysporum*, and *F. proliferatum* (Desjardins et al. 2000; Munkvold and Desjardins 1997; Musser and Plattner 1997; Seo et al. 1996). No other genera have been reported to produce fumonisins other than fungi. Production of fumonisins by *Alternaria* has also been reported (Mirocha et al. 1992; Mirocha et al. 1996). Some fumonisin-producing *Fusaria* have been known to produce AAL toxins, a compound with its structure closely related to fumonisins (Mirocha et al. 1992). The *Alternaria alternata*, a tomato pathogen, was found to produce AAL toxin (Wang et al. 1996). The fumonisins produced by *F. verticillioides* and *F. proliferatum* pose the greatest health hazard to human and animals since these species readily colonize corn all over the world (Bhatnagar et al. 2002; Dutton 1996; Proctor 2000; Riley et al. 1996; Scott 1993).

Nine structurally related fumonisins, including FB₁, FB₂, FB₃, FB₄, FA₁, and FA₂, have been identified. Fumonisin B₁ (FB₁) is the most abundant in maize. Chemically, FB₁ is a derivative (diester) of propane-1,2,3-tricarboxylic acid of 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane (Gelderblom et al. 1988; 1992a,b; Marasas 1995; Nelson et al. 1993; Riley et al. 1996; Scott 1993; Shier 1992). Other B series fumonisins such as fumonisin B₂ (FB₂), B₃ (FB₃), and (FB₄), occur at low levels. Related structurally to B series fumonisins, the four other series of fumonisins (A, AK, C, and P) also occur at low levels (Proctor 2000). The other fumonisins are resulted from removal of tricarballic acid and other ester groups (Musser et al. 1996; Seo et al. 1996).

Fumonisin has been shown to have diverse biological and toxicological effects. The mechanism of fumonisin toxicity is not well understood. Studies have shown that fumonisins inhibit ceramide synthase. Fumonisin has been shown to be hepatotoxic and carcinogenic in rats resulting in liver cirrhosis and hepatic nodules, adenofibrosis, hepatocellular carcinoma, ductular carcinoma, and cholangiocarcinoma (Gelderblom et al. 1988; 1991; 1992b; 1993; 1994; Marasas 1996). Studies on rats by Riley et al. (1996) suggested that fumonisin B₁ (FB₁) might act primarily as a tumor promoter. The tumor promoting activity of fumonisins has also been proposed by Huang et al. (1995), Wattenberg et al. (1996) to have been resulted from the stimulation or

suppression of signal transduction enzymes, the mitogen-activated protein kinase and protein kinase C. The mechanism of toxicological effect is a subject of intense study.

4.2 Genetics and Biosynthesis of Fumonisin

Genetic and biochemical studies on fumonisin biosynthesis are fairly recent. No comprehensive biosynthetic pathway and genetic and enzymatic establishments like aflatoxins or trichothecenes are available currently. Fumonisin biosynthesis has been of significant research interest in the last few years (Desjardins and Proctor 1999; Proctor et al. 1999a; Proctor 2000). A linear 19- or 20-carbon chain is the fumonisin backbone that is substituted at various positions with hydroxyl, methyl, and tricarballic acid moieties and an amino group at C-2 (Proctor 2000). Carbon labeling studies suggested that a PKS, alanine, methionine and other amino acids are involved in fumonisin synthesis (Desjardins et al. 1996a). Classical genetic analyses do provide some insight into the fumonisin biosynthesis and genetic control. Desjardins et al. (1996b) established that the genes responsible for fumonisin biosynthesis are closely linked on chromosome 1 when they studied on the FB₁-non-producing mutant of *G. fujikuroi* MP-A. By complementation analysis in FB₁-non-producing mutant of *G. fujikuroi* MP-A using *fum5*-containing cosmid clone, Proctor et al. (1999b) demonstrated that the *fum5* is involved in fumonisin biosynthesis. The predicted amino acid sequence of *fum5* showed high degree of similarity to fungal type I PKSs. Disruption and complementation analysis of the cloned *G. fujikuroi* gene, *fum5*, encoding a PKS, Proctor et al. (1999b) concluded that fumonisins are synthesized from polyketide rather than fatty acids. In order to identify additional genes that are potentially involved in fumonisin production near *fum5*, the DNA regions approximately 7-kb downstream and 15-kb upstream of this *fum5* gene have been sequenced (Proctor 2000). A total of 8 ORFs, 5 ORFs in the upstream and 3 ORFs in the downstream have been identified. Based on sequence homologies to known genes and proteins, four of the ORFs appear to encode proteins that would be expected to be involved in fumonisin production. One of these ORFs encodes a putative cytochrome P-450 monooxygenase. Even though none of the fumonisin biosynthetic genes was isolated except *fum5* for the PKS, classic genetic analysis using *Gibberella fujikuroi* (sexual stage of *F. moniliforme*) have identified four loci involved in fumonisin biosynthesis designated *fum1*, *fum2*, *fum3*, and *fum4* (Desjardins et al. 1995; Desjardins et al. 1996a,b; Plattner et al. 1996; Seo et al. 2001). Studies indicated that the allele, *fum1*, might be involved in regulation of fumonisin production (Plattner et al. 1996); the allele, *fum2*, might be involved in hydroxylation of fumonisin at C-10 position (Desjardins et al. 1996b); the allele, *fum3*, might be involved in hydroxylation of fumonisin at C-5 position (Desjardins et al. 1996b); an additional UV generated mutant (*uv26*) has similar phenotype like *fum3*

(Proctor et al. 1997). Xu and Leslie (1996) mapped *fum1* onto the chromosome 1 in *F. moniliforme* and the other *fum* loci were also mapped onto chromosome 1. Based on the recombination frequencies, a relative distance and linear relationship of the *fum* loci and some other gene loci have been established (Proctor 2000) to be in sequential order, *ald1*, *fum1*, *fum3* (*uv26*), *fum2*, and *OPA16*.

5 CONCLUSION

Within the last decade, significant advances have been made in mycotoxin research with respect to their identification, biochemistry, and genetics of their biosynthesis and regulation of toxin formation as well as mycotoxicosis and prevention and control. This is especially true for those mycotoxins such as aflatoxins, ST, fumonisins, and trichothecenes that are economically important in agriculture and pose greatest health hazard for human beings and livestock. The biosynthetic pathways, the clustering of these genes on the chromosome, the function of these genes and enzymes involved in the formation of these toxins have been elucidated in great details. Scientists have also acquired significant knowledge on the gene expression and regulation of toxin synthesis within the corresponding gene cluster. A better understanding of the nutritional and environmental factors that affect the production of these mycotoxins has been examined in greater details.

There remains, however, a vast gap in our understanding of the coordinated global regulation on toxin formation, the potential existence of signal transduction pathways underlying primary and secondary metabolisms, the effects of environmental factors, biotic and abiotic, on the toxin formation, the mode and regulation of plant-microbe (crop and fungi) interaction during infection. With the development and application of Expressed Sequence-Tag (EST) and microarray technologies, we will be able to study the whole organism on the molecular genetic level to address those unanswered questions. Only when we have a thorough and comprehensive understanding of the regulatory mechanisms of mycotoxin formation, will we be able to develop effective strategies to control mycotoxin contamination of food and feed on a consistent basis, resulting in a sustainable, nutritious, and healthy food supply for the entire increasing world population.

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Cellulose Degradation by Fungi

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1 INTRODUCTION

Cellulose and hemicellulose comprise about 50 and 30%, respectively, of the biomass the nature produces through photosynthesis and are by far the most abundant carbon polymers on earth. Lignin and minor amount of ash make up the remainder of the biomass. In addition to that which already exists in natural settings (especially in trees), cellulose/hemicellulose is a large component of wastes originating from municipal, agricultural, forestry, and certain industrial sources. Its abundance alone supports the contention that it is a valuable raw material for food production, energy generation, and chemical feedstock. Although only a small portion of what the nature produces is presently converted into useful products, this picture will likely change in the future, as the world population must ultimately depend on limited renewal resources for food, energy, and material goods (Millet et al. 1976). Except for directly burning wood and wastes for energy generation, most cellulose-derived products first require degrading of cellulose to constituent sugars before subsequent conversion, usually through microbial fermentation, of the sugars into a wide range of useful products, including ethanol, acetic acid, lactic acid, and antibiotics. Besides generating valuable chemical products, degradation of cellulose also reduces the waste disposal problems associated with landfills and burning forests (Katzen and Monceaux 1995). After several decades of research worldwide, there are two main routes to cellulose degradation: chemical (alkaline or acid) and biological (enzymatic or microbial). Of the latter route, fungi and anaerobic rumen bacteria are excellent candidates. In this chapter, we specifically focus on cellulose degradation by fungi and compare the economics of alternative processes.

2 BIOMASS AS A SUGAR SOURCE

Cellulose is a long linear polymer ranging from 1000 to 1000 000 D-glucose units. Glucose monomers are linked together with β -1,4-glycosidic bonds to form highly stable chains, and these chains further aggregates together via hydrogen bonds to form a rigid crystalline structure that is water-impermeable, water-insoluble, and resistant to enzymatic hydrolysis (Linko 1987). The boundary and sequence structure of the molecules determine the chemical properties of cellulose (Alen and Sjostrom 1985). On the other hand, hemicellulose, which is alkali-soluble, is composed of short, highly branched copolymer of both six-carbon and five-carbon sugars. The branched structure allows hemicellulose to exist in an amorphous form that is more susceptible to hydrolysis. Compared to cellulose, which is similar across all biomass sources, hemicellulose is quite diverse in structure and composition, depending on the source. The hydrolysis product of hemicellulose is typically a mixture of xylose, arabinose, galactose, mannose, glucuronic acid, and other sugars. The ratios of five-carbon and six-carbon sugars depend on the source species of the biomass. Compared to hardwoods and agricultural residues, softwoods generally contain more six-carbon sugars (D-glucose, D-galactose, and D-mannose) but less five-carbon sugars (D-xylose and L-arabinose) (Alen and Sjostrom 1985). With cellulose, a chief engineering challenge is degradation; whereas, with hemicellulose, a major limitation lies in microbial fermentation of some sugars. Although there exists significant variations in the composition among species, biomass roughly consists of 50% cellulose, 30% hemicellulose, and 20% lignin.

Cellulose material obtained from waste sources is typically inexpensive but difficult to degrade. Certain

processes require that lignin and other components will first be removed from cellulose before the actual processing of the material can proceed. In order to remove this nonbiodegradable component and other impurities from cellulose feedstock, we must pretreat the feedstock with mechanical, thermal, or enzymatic means. Of the many possible products derived from cellulose, ethanol manufactured via microbial fermentation has long captured the attention of many researchers in the following areas of applications: (a) potable ethanol for beer, wine, and distilled beverages, (b) solvent ethanol for laboratory and pharmaceutical applications, and (c) as a fuel additive/blend to reduce harmful emissions or as a complete substitute for gasoline. Lactic acid, which is a specialty chemical utilized mainly in the food industry as an acidulent and preservative, finds use in the chemical processing industry as a metal deliming agent (Iyer and Lee 1999; Schmidt and Padukone 1997).

A number of processes have been developed to degrade cellulose and to produce ethanol, although successful commercial units to date remain few in number. The most well known is simultaneous saccharification and fermentation (SSF), where the bioreactor brings together cellulose, glucose, cellobiose, cellulase enzymes, yeast (but not fungi), and nutrients to produce ethanol (Philippidis 1992). This process involves a number of different steps all carried out in one vessel. To avoid the setback associated with SSF, another process known as *mixed culture and fermentation* was developed, where a consortium of fungi and bacteria, or fungi and yeast, rather than simply yeast alone as was in SSF, convert cellulose into ethanol in one combined step (Wilke et al. 1983). We will review cellulose pretreatment and focus on fungal conversion of cellulose into ethanol, and finally we will touch upon the economic aspects of ethanol production.

2.1 Fungal Strains

Since the early days of civilization, humans have been practicing converting hexose to ethanol with yeast, specifically *Saccharomyces* species for alcoholic beverages. Thus, it is only natural that this process technology is proven and quite advanced. More recently, many studies have focused on utilizing bacteria, especially *Zymomonas mobilis*, to produce industrial ethanol from hexose where taste is not at all a factor. In comparison, converting pentose and other sugars to ethanol is a much more recent development and still faces many unresolved challenges.

The most intensively studied fungus capable of cellulose degradation is *Trichoderma reesei*, which was originally named *T. viride* but later renamed in honor of a pioneer in cellulase research, E. T. Reese. This fungus prodigiously produces an excellent ensemble of three major classes of cellulase enzymes that function synergistically to convert cellulose all the way to glucose: (a) endoglucanases, which attack at random locations in the cellulose chain, (b) exoglucanases or glucanhydrolases, which liberate glucose dimer cellobiose from the end of the cellulose chain, and (c)

β -glucosidases, which release constituent glucose monomers from cellobiose and soluble celloextrins. Many less suitable fungi also produce cellulases, but not necessarily in an optimal ensemble. Although *T. reesei* is efficient in degrading cellulose, it alone cannot convert cellulose directly to a useful final product, unless glucose happens to be the target product. In fact, only a few filamentous fungi are known to convert cellulose efficiently into useful final products. The following are among the few filamentous fungi that are capable of hydrolyzing cellulose as well as converting the sugars liberated from hydrolysis to ethanol or acetic acid: *Monilia*, *Neurospora crassa*, and *Fusarium oxysporum*; the following fungal species are capable of converting xylose to ethanol: *Fusarium*, *Mucor*, and *Paecilomyces* (Singh et al. 1992). The disadvantage of direct conversion is the slow conversion rate compared to yeast, although conversion efficiency can be as high as yeast (Krishna et al. 2001). Thus, economic considerations generally preclude converting cellulose to ethanol with these fungi. Technical feasibility does not translate to economic feasibility. Table 1 summarizes the different types of fungi responsible for converting cellulose to either ethanol or acetic acid.

2.2 Cultivation Conditions and Nutrient Requirements

2.2.1 Carbon Source

Since, substrate represents a major cost item (estimated to be anything from 30 to 70%), waste will not be tolerated if the process is to have a chance to be economically viable. Since D-xylose is a major component of cellulosic biomass and since few organisms can convert it at all—and those who do usually do so inefficiently, the cellulolytic strains that can convert D-xylose to ethanol represent a great opportunity (Schneider 1989), and they should be scrutinized further in future studies. Especially notable are the cellulolytic strains of *Monilia*, which are capable of producing ethanol from both D-glucose and D-xylose. In a study, this fungus converted more than 40% of D-xylose into ethanol in 7 days (Kumar

Table 1 Fungi responsible for cellulose degradation

Organism	Features
<i>F. oxysporum</i> F3	Direct conversion of cellulose into ethanol
<i>F. oxysporum</i> DSM 841	Efficient direct conversion of waste cellulose to acetic acid
<i>Monilia</i> sp.	Direct conversion of cellulose to ethanol
<i>N. crassa</i>	Direct conversion of cellulose/hemicellulose into ethanol

Source: Singh et al. (1992).

et al. 1991). Like *Monilia*, the fungus *F. oxysporum* also successfully converts a variety of carbon sources: glucose, xylose, cellobiose, with a yield of 5.0–9.1 g/l of ethanol. *N. crassa* is yet another species that biologically transforms cellulose all the way to ethanol (Singh et al. 1992). It should be noted that many fungal cellulose degradation studies and fungal cellulase production studies employ well-defined model substrates such as carboxymethyl cellulose (CMC), Avicel (which is more crystalline), or xylan. The synthetic media are further supplemented with minerals and yeast extract. Although defined media tend to yield more reproducible laboratory results, they do not realistically approximate commercial processes. On a related point, these model compounds also serve as substrates for standardized cellulase activity measurements. For example, a common gauge of endoglucanase activity is decreased CMC viscosity. Note that a decrease in the CMC viscosity does not linearly correspond to an increase in the reducing sugar level.

2.2.2 Nitrogen Source

Nitrogen is a major cellular constituent of any organism, and fungi are certainly no exception. Any successful media formulation must carefully balance microorganism's inherent need for nitrogen. Nitrogen limitation usually leads to slow or no growth or even death of fungi, slow xylose consumption, and retarded ethanol production. Table 2 summarizes the different sources of nitrogen and the typical concentration levels present in fungal growth media in a laboratory setting. In a defined media formulation, typical nitrogen sources are ammonia, nitrates, and urea. In complex media, peptone, tryptone, yeast extract, wheat bran, and an array of protein digests supply other crucial but less understood set of nutrients. Metabolic pathways trace the fate of carbon rather than nitrogen sources. Nonetheless, nitrogen sources' effect on fungal metabolism can be pronounced. For example, Sale (1967) reports that ammonium ions stimulate glycolysis by counteracting ATP's inhibition of phosphofructokinase, and they stimulate pentose phosphate pathway by derepressing glucose-6-phosphate dehydrogenase. However, the effects of nitrogen sources are generally not as well understood. From

Table 2 Nitrogen sources in lignocellulosic conversion processes by fungi

Nitrogenous compounds	Concentration (g/l)
Ammonium nitrate	1.0
Ammonium dihydrogen phosphate	2.0
Potassium nitrate	2.5
Sodium nitrate	2.0
Urea	2.0
Peptone	5.0
Yeast extract	0.25
Potato protein liquor	40

Source: Singh et al. (1992).

economic feasibility viewpoint, supplementing nitrogen from complex sources is undesirable because the practice adds significantly to the cost. A future challenge is to eliminate completely the need for nitrogen addition and to satisfy fungi's nitrogen demand entirely from natural lignocellulosic sources. At least, one would consider cheaper sources of nitrogen.

2.2.3 Minerals and Vitamins

Minerals, trace elements, vitamins, and growth factors play a vital role in the growth of fungi and their biosynthesis of metabolites. There has been no systematic study on the effect of minerals and trace elements such as Ca, Mg, Fe, Zn, Cu, Co, and Mn on growth of fungi and ethanol production by fungi. The present strategy is to provide sufficient quantity of these compounds, estimated based on information gathered from other microbial fermentation, such that they do not become rate limiting. However, it is unclear what constitutes an optimal level. The large number of compounds involved immediately translates to impractical number of runs. For example, if one were to conduct brute-force experiments by varying one variable at a time, say, each variable set at three separate levels (high, medium, and low), n variables yields 3^n combinations if the variables interact with one another, or $3n$ combinations if the variables do not interact. To appreciate the magnitude of this undertaking, 10 trace compounds translates to $3^{10} = \sim 300,000$ possible combinations! And that does not count the unsuccessful runs. Although a sound experimental design can reduce the number of runs, the number of runs required remains utterly impractical if the variables interact.

2.2.4 Aeration

Under aerobic conditions, the fungi listed in Table 1 grow well but are unable to produce ethanol. On the other hand, under anaerobic or microaerobic conditions, they are able to produce ethanol but grow only slowly. Thus, if one is to pursue cellulose-to-ethanol with a single fungal species, a common strategy is to cultivate fungi first under aerobic conditions, followed by ethanol production under anaerobic conditions. For example, in a study utilizing *N. crassa*, the mold grew first aerobically for 48 h. Subsequently, the content of the growth flask was transferred to a special flask that had a capillary opening at the top to exclude oxygen from entering while allowing carbon dioxide to escape as ethanol is produced (Singh et al. 1992).

Compared to higher organisms, fungi are more susceptible to changes in their surrounding environments. However, the cytoplasmic pH of the fungi tends to change very little over a wide range of extracellular pH. In most prior studies, which have been conducted without active pH control, the initial pH values are within the range of 5–6. Both the rate of cellulose degradation and product distribution depend on the pH. For example, Enari and Shihko (1984) report an optimal pH of 5.5 for ethanol production for *F. oxysporum*, but acetic acid

production increased considerably at pH 6.0. For *N. crassa*, maximum ethanol production occurred in the range of 5.0–6.0, which coincided with maximum cellulase activity (Deshpande et al. 1986).

In nature, fungi may be exposed to different environmental conditions that can inhibit their growth. In general, the intrinsic enzyme (including cellulase) reaction rate and its deactivation rate both increase with temperature. As temperature increases, the rate of enzyme deactivation surpasses that of enzyme reaction temperature; thus, the apparent enzyme reaction rate exhibits a maximum with respect to temperature. Deshpande et al. (1986) show that *N. crassa* works best at 37°C. It converts more than 90% of cellulose into ethanol within 4 days at 37°C. However, above this optimum, the cellulase activity increases but the production of end product, such as ethanol, decreases. Table 3 shows optimum pH and temperature needed for the conversion of cellulose by different fungi.

Light plays an important role in the growth of filamentous fungi. It can hinder or arouse the emergence of mycelia, and sensitivity to light is species dependent. In *Trichoderma*, the most efficient wavelengths to induce growth are 310, 430, 455, and 480 nm.

3 DEGRADATION OF COMPLEX MOLECULES

3.1 Cellulose Degradation

In a simplistic summary, there are four major steps in generating bioethanol and other biomass-derived products: (a) generate biomass through photosynthesis, (b) process raw biomass to a form suitable for microbial fermentation, (c) ferment biomass into ethanol (or other products), and (d) recover product and recycle unfermented residual biomass. We will not dwell here on the first step that takes place in nature. We present the remaining steps to place in proper perspective fungi based cellulose degradation. Pretreatment of the cellulosic biomass is necessary in the yeast and bacteria based cellulose conversion, because these microorganisms do not easily hydrolyze woods and agricultural residues. If they did, biomass material would not remain for long nor accumulate (and wood would be totally unsuitable as a

building material). In order for organisms to digest biomass efficiently and productively, it must be pretreated by mechanical, chemical, or biological means to break down the stable polymeric structure of cellulose, hemicellulose, and lignin. For this purpose, there are presently four major upstream processing technology platforms, and many variations exist around these basic technologies: (a) concentrated acid hydrolysis carried out at low temperature, (b) dilute acid hydrolysis carried out at high temperature, (c) enzymatic (cellulase) hydrolysis, and (d) biomass gasification (pyrolysis). The two acid technologies are old developments; whereas, the enzymatic process is relatively newer, and the gasification process is much more recent. Enzymatic hydrolysis alone is normally insufficient in degrading cellulose; thus, mechanical and chemical pretreatment becomes necessary. Finally, the fermentation step normally is further subdivided into two steps: (a) conversion of cellulose to glucose and (b) conversion of glucose to ethanol (Blazej and Kosik 1985). A separate organism is usually responsible for each one of these two steps. Only a few microorganisms in nature can perform tasks in both steps.

Physical pretreatments can be either mechanical or nonmechanical (Fan et al. 1987). In the mechanical process, the force applied in pretreatment breaks lignocellulosic materials into small highly digestible particles for enzymatic hydrolysis, where soluble enzyme works on insoluble cellulose mainly at the liquid–solid interface and the observed reaction rate is not necessarily limited by the intrinsic reaction rate but by mass transfer. Thus, breaking down particles increases the surface area for reaction. In addition, the nonmechanical pretreatment with high temperature steam causes disruption in the fundamental molecular and crystalline structure of the cellulose.

Ball Milling: Ball milling is one of the best methods to enhance enzymatic hydrolysis. In this process, shear and mechanical forces break down the particle size in order to facilitate the digestion of cellulose. Ball milling, although highly efficient, cannot be operated on a large scale because of the high cost and the lengthy time it takes to break down the molecular structure of cellulose. Like ball milling, two-roll milling also decreases the particle size.

To facilitate digestion, chemical agents such as alkali or acid solutions have long been utilized to pretreat cellulose. Acid treatment increases the reduction power of the reactive groups along the cellulose polymer chain, and cellulose is thus destabilized and rendered more susceptible to subsequent attacks (Fan et al. 1987). Concentrated acid disrupts the hydrogen bonds between cellulose chains, thus, it breaks the otherwise highly stable crystalline structure of cellulose and dissolves cellulose into a thick, hydrogel-like solution. Cellulose is now in an amorphous state and is readily digested. Addition of water dilutes the acid and rapidly breaks the β -1,4-glucosidic linkage, leading to complete hydrolysis of cellulose to glucose (known as saccharification) with an yield that is close to the theoretical value. At the end of saccharification, glucose molecules remain intact. Concentrated sulfuric acid is the acid of choice, although other acids

Table 3 Optimum pH and temperature range

Organism	Optimum pH	Optimum temperature (°C)
<i>F. oxysporum</i>	5.5	30
<i>F. oxysporum</i>	5.0	30
<i>F. oxysporum</i>	6	34
<i>Monilia</i> sp.	—	26
<i>Mucor</i> sp.	5.4	30
<i>N. crassa</i>	5.0–6.0	28–37

Source: Singh et al. (1992).

(hydrochloric, nitric, and phosphoric) are also employed in some laboratory studies. In a typical process, five parts of ~75% sulfuric acid is added to four parts of dried biomass containing ~10% moisture at 50°C. This is followed by diluting with water to ~25% acid and holding at 100°C for 1 h. The following schematic shows the fate of cellulose in concentrated acid.

cellulose → acid complex → oligosaccharides → glucose

High temperature dilute acid process works in two stages, with the first stage optimized for the more readily hydrolyzed hemicellulose and the second stage's conditions specifically tuned for the tougher cellulose. Typical conditions are 0.7% sulfuric acid at 190°C for the first stage and 0.4% at 215°C for the second stage. The residence time for each stage is approximately 3 min. A dilute acid process exposes cellulose to acid for a shorter time compared to a concentrated acid process, but only about 50% of the glucose is recovered in a dilute acid process compared to nearly 100% recovery for a concentrated acid process. In hot dilute acids, hydrolysis of cellulose proceeds as follows.

native cellulose → stable cellulose (hydrocellulose)

→ soluble polysaccharides → glucose

Under certain conditions, alkaline solutions can degrade cellulose by breaking down its long molecular chain at random interchain locations and generate a number of shorter molecules of cellulose. Many alkalis such as metal hydroxides, salts in a strong alkali solution, inorganic salts, a number of amines, and related compounds break the hydrogen bonds between cellulose molecules. Alkalis commonly employed in practice include sodium hydroxide and ammonia. The cellulose structure resulting from alkaline treatment is more amorphous. The swollen cellulose structure allows subsequent enzymatic degradation to proceed more readily because enzyme molecules can gain access to its interior region. Unlike acid treatment, alkaline treatment alone is usually insufficient in releasing constituent monomer sugars to theoretical yield. As a result, it is normally only a

pretreatment step that is followed by an enzyme step. Table 4 shows the sugar yields from different alkali pretreated biomass sources after enzymatic digestion.

Biological degradation of cellulose features prominently in the nature's carbon cycle. Cellulase refers generally to the enzymes that degrade cellulose. Most known cellulases are of fungal origin. Instead of simply one enzyme, cellulase from most fungi is actually a collection of several distinct enzymes that work synergistically to accomplish the overall task of cellulose degradation. We can classify cellulase-secreting fungi into different groups: brown rots, white rots, and red rots. Unlike white and red rots that degrade cellulose and lignin, brown rots degrade mainly cellulose (Kirk 1976).

Several microorganisms are known to produce cellulase that hydrolyzes cellulose and hemicellulose. The best known is the filamentous fungi *T. reesei*. This microorganism generates at least three well-known enzymes known as: (a) β -1,4-D-glucan glucanohydrolase (an endo-enzyme), (b) β -1,4-D-glucan cellobiohydrolase (an exo-enzyme), and (c) β -glucosidase (Jeffries 1987). The most common cellulase, β -1,4-D-glucan glucanohydrolase or C_{x} , has several constituents. One of these constituents acts as the initiator of cellulose hydrolysis. The second enzyme, β -1,4-D glucan cellobiohydrolase, has two constituents: β -1,4-glucan glucanohydrolase excises one glucose molecule from the nonreducing end of the cellulose chain, and β -1,4-glucan cellobiohydrolase removes two glucose units at a time from the end of the cellulose chain. It is further divided into two subcomponents CBHI and CBHII. The third class of enzyme, β -glucosidase, does not act directly on cellulose. It is nonetheless important because it hydrolyzes the glucose dimer cellobiose, which yeast cannot ferment, to liberate two glucose monomers, which yeast can readily utilize. Furthermore, cellobiose inhibits cellulase enzymes much more strongly than glucose does. The presence of sufficient quantities of β -glucosidase helps reduce feedback product inhibition.

Through pyrolysis, biomass is gasified into synthesis gas. Above 300°C, cellulose is degraded into volatile, gaseous products such as hydrogen, carbon monoxide, and carbon dioxide. At intermediate temperatures, decomposition is

Table 4 Yield of sugars from enzyme hydrolysis (5 wt% suspension) of acid and base treated solid based on 100 lb of original material

Material	Glucose	Poly glucose	Xylanose	Arabinose	Conversion of available carbohydrate		
					G & PG	Pentose	Sugar
Barley	16	2.4	0.21	0.06	47.7	2.2	36.7
Corn stover	13.8	8.1	0.93	0.07	66.4	35.7	64
Cotton gin trash	6.9	1.8	0.32	0.05	44.8	6.1	35.6
Rice hulls	7.5	1.2	0.01	0.01	27.2	0.3	21.7
Rice straw	15.6	8.3	0.55	0.16	66.9	15.8	60.8
Sorghum straw	13.9	9.7	1.8	0.23	80.8	22.8	67.3
Wheat straw	14.6	3.5	2.07	0.10	57.5	36.5	53.4

Source: Wilkes et al. (1983).

rather slow and the products formed are less volatile. Atmospheric conditions greatly affect pyrolysis. In the presence of oxygen, dehydrogenation and depolymerization occur quickly. On the other hand, in the presence of inert substances, depolymerization is slow, and unwanted byproducts appear (Wilke et al. 1983). In subsequently steps, the synthesis gas is bubbled into a submerged culture, and anaerobic microorganisms (e.g., bacteria *Clostridium ljungdahlii*) convert the pyrolysis product into ethanol (Klason et al. 1990). The chief obstacle of this technology is the high cost of energy (mainly electricity) to heat the biomass, and the low value of the synthesis gas thus produced. Pyrolysis treatment may be economically viable if the fermentation product is valuable.

3.2 Simultaneous Saccharification and Fermentation

In late 1970s, Gulf Oil Company developed a process for simultaneous saccharification of cellulose and fermentation of the hydrolysis product to ethanol (Katzen and Monceaux 1995). Researchers at the University of Arkansas later improved upon the SSF process, summarized in Figure 1. Conceptually, we simply replace the acid hydrolysis step with an enzymatic one and carry out the combined operations of hydrolysis and fermentation in a single reactor to convert biomass into ethanol.

Most reports on the SSF process cite combining two separate reactors for saccharification and fermentation into one reactor as an advantage; however, this claim is misleading. Perhaps because the researchers may have simply obtained the cellulase enzyme off the shelf for their studies, they ignore the fact that the enzyme has to be produced separately in an economical fashion (Shin et al. 2000). In practice, there is an additional cellulase production step, which is nontrivial from the operational and economic

viewpoint. Fermenting biomass with the fungus *T. reesei* to produce the cellulase needed in SSF is perhaps just as complex and expensive an operation as the cellulose-to-ethanol conversion portion.

3.2.1 Process Description

We give the description of a typical SSF process that aims to recycle solid waste into ethanol, which functions as an alternative fuel. The solid utilized in this process is approximately two-thirds urban waste and one-third pulp mill waste. This solid mixture contains approximately 57% in cellulose. The waste is pretreated, sterilized, and then forwarded to three reactors of 2500 gal each. A culture of mutant fungus *T. reesei* is inoculated into each reactor. The fungus continuously produces a full complement of cellulases that degrade cellulose. The total residence time for each cellulase production strain is 48 h. Ninety percent of the cellulose introduced into the reactors is degraded into sugars, such as pentose, xylose, arabinose, and glucose. Subsequently, the degraded cellulose is cooled in a heat exchanger and sent into 12 reactors for fermentation into ethanol. In this system, one can shut down four reactors while continuing operating the remaining reactors in full swing. The four reactors have an effective residence time of 48 h and are maintained at 40°C. The initial feed to the reactors contains 8% of intact cellulose, and the remaining 92% is degraded cellulose, which is hydrolyzed to glucose. The yeast simultaneously ferments the glucose generated to produce 2–3.6 w/v% ethanol beer slurry. Any unconverted material is recycled back to the process.

In Szczodrak's study (1989), the filamentous fungi *T. reesei* produced the cellulase, and a thermotolerant yeast strain *Kluyveromyces fragilis* FT 23 carried out the conversion of sugars to ethanol. Table 5 gives a summary of the percentage of ethanol produced.

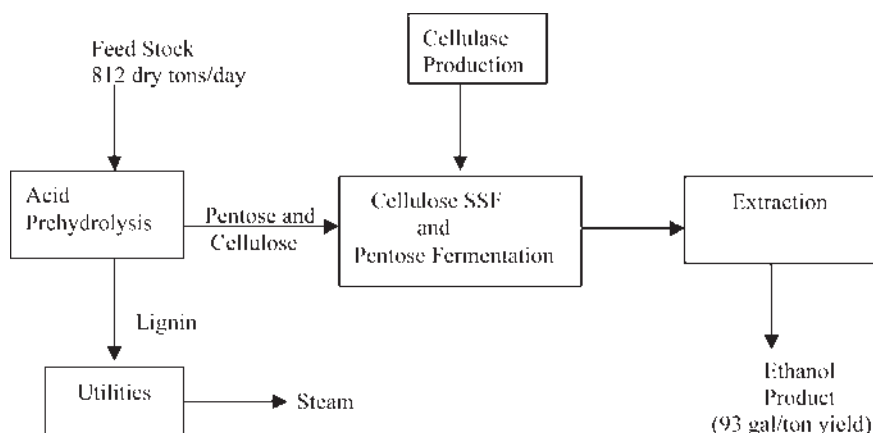


Figure 1 The SSF process flow chart. From So and Brown (1999).

Table 5 The SSF of chemically modified straw by *T. reesei* cellulase preparations derived from the parent and β GDase mutant strain and *K. fragilis* cells

Cellulase source	Activity (U/g straw)		Time (h)	Ethanol percent (w/v)	Glucose hydrolyzate (mg)	Final pH
	Cellulase	β GDase				
<i>T. reesei</i> F-522 (parent)	40	14.1	24	1.9	0	5.0
			48	2.5	0	4.9
<i>T. reesei</i> F-522-V-7 (mutant)	40	64.2	24	3.4	—	4.7
			48	3.4	0	4.7

Source: Szczodrak (1989). One unit of enzyme activity (U) is defined as the amount of the enzyme that liberates one moles of reducing sugars (calculated as glucose) per minute under the assay conditions. β GDase stands for endo β -1,4-D-glucanase.

3.2.2 Mixed Cultures and Fermentation

One disadvantage of the SSF process is the number of steps because of the separate steps needed for cellulase generation. In the original SSF process, cellulase, yeast, cellulose, and other nutrient supplements are all thrown into one bioreactor. To reduce the number of steps and cost, a modified process termed mixed cultures and fermentation has been developed. In this process, more than one organism coexist in the same reactor. While one organism degrades cellulose into sugars, another ferments sugars into ethanol, acetic acid, and lactic acid. This process is more efficient because it eliminates the steps associated with the separation of the enzyme from the products. In the process shown in Figure 2, *C. thermocellum* saccharifies cellulose into glucose and cellobiose; the same organism also hydrolyzes hemicellulose to xylose and xylobiose. Furthermore, it ferments glucose and cellobiose to ethanol, acetic acid, and lactic acid. The second organism *C. thermosaccharolyticum* cannot degrade cellulose, but it ferments glucose, cellobiose, xylose, and xylobiose to ethanol, acetic acid, and lactic acid (Wilke et al. 1983). Mixed culture fermentation is performed under anaerobic conditions, at a temperature of 60°C and pH 7. In summary, *C. thermocellum* efficiently degrades cellulose and hemicellulose, and *C. thermosaccharolyticum* then metabolizes xylose to generate ethanol and useful byproducts.

4 ECONOMIC ASPECTS OF ETHANOL PRODUCTION

Ethanol has been used as a fuel in the United States since the beginning of the 20th century. However, it was quickly

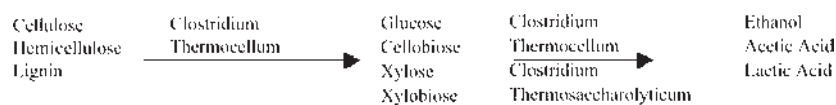
replaced by lower priced gasoline. With the oil crises in the 1970s and increasing environmental concerns, ethanol has regained some appeal as an alternative motor fuel (Linko 1987) or as an additive to be blended with gasoline. Because ethanol is an oxygenate that reduces vehicle exhaust emissions, it is environmentally friendly (Bollok et al. 2000). To remain successful in that role, the cost of production must be low compared to gasoline. In this part, the economic aspect of ethanol production will be discussed.

4.1 Economic Analysis of Ethanol Conversion Technologies

Several technologies have been developed in order to convert efficiently cellulose to ethanol. The best-known processes that we discuss in the earlier section are: (a) SSF, (b) dilute sulfuric acid hydrolysis and fermentation, and (c) fast pyrolysis and fermentation. We will briefly describe the dilute sulfuric acid hydrolysis process and the fast pyrolysis process, and then compare the cost and the production of ethanol among the three processes.

Figure 3 illustrates the process of ethanol production by dilute sulfuric acid hydrolysis and fermentation. In the hydrolysis step, cellulose is pretreated in 0.05 g/l of sulfuric acid at 180°C. For the purpose of economic comparison, we consider the concentration of sugars yielded to be 103.7 g/l. Following hydrolysis, a strain of fungus is responsible for the continuous fermentation of sugars (pentose and hexose) into ethanol (So and Brown 1999).

Figure 4 shows the Waterloo fast pyrolysis and fermentation process. Its pretreatment step uses 5% hot sulfuric acid. In the fermentation step, fungi degrade cellulose

**Figure 2** Mixed culture hydrolysis and fermentation.

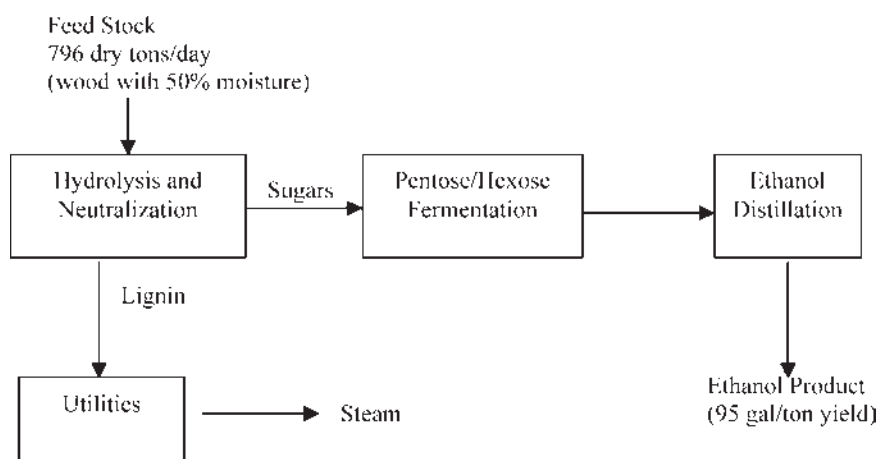


Figure 3 Dilute sulfuric-acid hydrolysis and fermentation process. From So and Brown (1999).

into sugars while bacteria ferment the different types of sugars (pentose and hexose) to ethanol.

The capital cost analysis given in Table 6 shows that fast hydrolysis, SSF, and acid hydrolysis all have fairly similar total capital costs; the small differences are probably within the error of estimates. Likewise, the operating cost analysis presented in Table 7 also shows similar numbers, albeit the cost of ethanol is the lowest with the SSF process.

4.2 Costs and Benefits of Ethanol Production

The production cost of ethanol must be low for it to be competitive with other existing fuels on the market. Presently, the competition comes from methyl tertiary butyl ether (MTBE) in gasoline blend, but in time the target for comparison will shift. Fluctuation in the prices of other energy sources makes it difficult to predict the future of

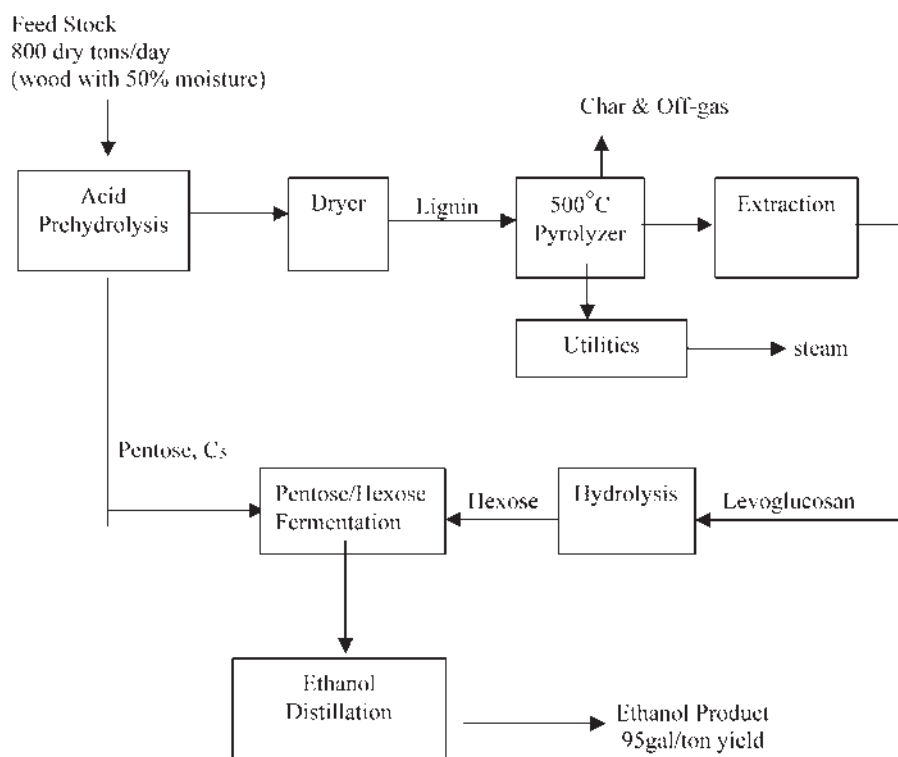


Figure 4 Waterloo fast pyrolysis and fermentation process. From So and Brown (1999).

Table 6 Capital cost investment comparisons

Fast pyrolysis		SSF		Acid hydrolysis	
Plant areas	Capital cost (millions dollars)	Plant areas	Capital cost (millions dollars)	Plant areas	Capital cost (millions dollars)
WFPP	12	Pretreatment	16	Hydrolysis	2
Fermentor	22	Pentose fermentor	4	Fermentor	27
		Cellulase production	2		
		SSF	16		
Ethanol recovery	11	Ethanol recovery	3	Ethanol recovery	14
Utilities	12	Utilities	12	Utilities	12
Off-site tankage	3	Off-site tankage	3	Off-site tankage	3
Fixed capital	60	Fixed capital	56	Fixed capital	58
Working capital	9	Working capital	8	Working capital	9
Total capital	69	Total capital	64	Total capital	67

Source: So and Brown (1999).

bioethanol. Although from time to time the government may provide tax incentives to jump-start an interest in renewable energy resources—which in practice is synonymous with ethanol from biomass, the production process must be inherently competitive for it to be sustained in the long run. Table 8 shows a study of ethanol production in California. It gives the price of different feedstocks at near-term and mid-term operation at a large scale. The target price takes into account the operating costs, the debt, and return on investment. The target price decreases from near-term to mid-term, as the technology improves and forces down the production cost. Even when the cellulosic feedstock is inexpensive, conversion into ethanol may be costly. Cellulase enzymes cost 45 cents/gal of ethanol and are, therefore, too expensive at the commercial level.

The critical factors that contribute to the cost of ethanol are summarized below.

(a) The cost of collected feedstock such as municipal and commercial wastes must be low. Thus, the production facilities should be located near the source of biomass material. Furthermore, we can cross the break-even point easier with higher cellulose content in the municipal and commercial wastes. (b) Ideally, the production site should be close to the consumption site to minimize shipping. Ethanol absorbs moisture very quickly and attacks rust spots in the transportation system. This attack provokes corrosion along the distribution system. (c) Because feedstock constitutes a major fraction of the overall cost, we must efficiently utilize not just cellulose but all components in the feedstock, especially xylose and lignin. This is especially true for

Table 7 Operating cost comparison with cost figures expressed in millions of 1997 US\$

	Fast pyrolysis	SSF	Acid hydrolysis
Total capital	69	64	67
Annual operating cost			
Wood	11.09	11.25	11.03
Steam	1.15	−4.50	−2.95
Electricity	1.20	1.37	1.25
Operating labor	0.27	0.58	0.30
Supervisory	0.04	0.09	0.05
Maintenance and repair	3.52	3.36	3.48
Indirect operating costs ^a	3.67	3.64	3.58
General expenses ^b	4.40	3.49	3.63
Annual capital Charge	13.80	12.80	1334
Total annual operating costs	39.21	32.09	33.70
Production cost of ethanol (\$/gal)	1.57	1.28	1.35

Source: So and Brown (1999).

^a Indirect operating costs include overhead, local taxes, and insurance.^b General expenses include administrative, distribution, selling, research, and developments costs.

Table 8 Assumed feedstock cost, ethanol production yields, ethanol prices from cellulose based biomass

Time frame	Near-term	Mid-term
Feedstock price (\$/BDT)		
Forest material	36	38
Agricultural residue	24	26
Waste paper	− 10	− 10
Ethanol yield (gal/BDT)		
Forest material	69.3	77.4
Agricultural material	62.4	64
Waste paper	74.4	81.7
Target ethanol price (\$/gal)		
Forest material	1.73	1.23
Agricultural residue	1.69	1.24
Waste paper	1.64	1.39

Source: California Energy Commission 2001.

feedstock derived from forest and agricultural residues. Furthermore, unutilized residues incur a disposal cost. Strain improvement and recycling of residuals move us closer toward fulfilling this goal. (d) For processes that depend on fungi-derived cellulase for cellulose degradation, the cost of the enzyme must be dramatically reduced. Other commercially viable processes that utilize cellulase (e.g., stone-washed jean in textile processing) deal with high-valued products and require only partial hydrolysis. In contrast, ethanol production from cellulose deals with bulk commodity products and requires nearly complete hydrolysis. Thus, relative to the price of the product, the cost of cellulase looms high. Advances in biotechnology and genetically engineered organisms offer possible solutions to lower prices for the enzymes. (e) For processes that utilize acid for cellulose degradation, acid poses a significant cost. Thus, recovering acid and separating sugars efficiently and inexpensively become critical. Advances in separation and membrane technologies should help.

There are several major issues that reach beyond the simple economics in evaluating the future of bioethanol. From the national security standpoint, heavy dependence on imported oil restricts our options in dealing with international matters. Bioethanol should be an integral part of an overall picture showing other forms of energy: natural gas, coal, nuclear, hydroelectric, and, of course, oil. Energy diversity and flexibility is a prudent course that the government should be encouraged to take. Energy plays a central role in the economic health of a society. Finally, production of ethanol from biomass impacts both positively and negatively on the environment. Thinning forests to reduce the risk of wildfires generates biomass as the feedstock to ethanol production. By reducing the number of trees, the severity of wildfires will be reduced; so will the concomitant emission of hydrocarbons and carbon monoxide generated by the fires. When blended with gasoline, ethanol reduces the various harmful emissions:

CO, NO_x, and volatile organic carbons. We also envision a future where biomass-derived products (ethanol, acetic acid, acetone, etc.) become significant raw materials for chemical processing industries (California Energy Commission 2001).

5 CONCLUSION

The most important commercial product of cellulose degradation is ethanol, which has the potential of filling the national energy and raw material needs. Furthermore, burning bioethanol has the advantage that it does not contribute to green gas emission from the viewpoint of overall carbon cycle. The present technology derives carbon mainly from corn in a two-step process: hydrolysis of starch to sugar followed by fermentation of sugar to ethanol. In the future, starch is perhaps better saved for food or chemical raw material. To make the process competitive against existing sources of energy and raw materials, we must expand the feedstock to include lignocellulosic biomass. Economics play a major role in deciding which process is ultimately commercially viable. To this end, future research must focus on minimizing unfermented residual biomass, especially lignin and hemicellulose. We must attack an old problem systematically with a multipronged approach and with newly discovered tools in biotechnology: genetic engineering, site directed mutagenesis, and metabolic engineering.

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The Importance of Wood-Decay Fungi in Forest Ecosystems

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1 INTRODUCTION

This chapter considers the role of wood-decay fungi in forest ecosystems in the broadest sense, embracing examples of fungi displaying wood and litter degradative activities, even if only transiently. The aim is to emphasize the fundamental significance and pervasiveness of wood-decay activity as an integral process in ecosystem functioning. I therefore present a coherent text of diverse themes, but not a comprehensive treatise of any. Each individual topic presented offers an overview of material that has formed the basis of many individual and extensive texts. I have therefore been selective, have tried to avoid lists of species and instead attempted to highlight important ecological activities, features, trends, and concepts. In examining the current status of knowledge, I have on occasion restated familiar works, thus providing progression to more recent research. As a result of my own background and interests examples are mostly drawn from the Northern Hemisphere and information regarding, for example, important tropical biomes, has largely been omitted. The chapter aims to identify some general concepts underpinning wood-decay fungal ecology, and provide guided access to pertinent literature where possible. I conclude by identifying neglected arenas of study and speculating on future research trends. After-all, continued investigation concerned with wood-decay fungi and their role in forest ecosystems is an academically challenging and immensely important process in biological, ecological as well as practical terms.

2 WOODLAND ECOSYSTEMS AS A GLOBAL RESOURCE

Woodland ecosystems are a major planetary resource, involving about a third of the land surface. Forests and

woodlands provide a habitat for biological diversity. They contribute substantially to the global carbon cycle and are the natural venue for many derived pharmaceutical, practical, and edible products. Woodlands are also an environment for indigenous peoples, and provide much aesthetic and amenity value. Alarming, our forests are being lost at a rate of around 14.6×10^6 ha/yr due to pollution, disease, and excessive exploitation (Anon 2001).

Wood accounts for around 80% of the global organic carbon reservoir. About 70% of woodland net primary production is deposited annually as litter, whilst the remaining 30% accumulates at least transiently as woody biomass (Boddy 1991; Rayner and Boddy 1988). Below ground, persistent lignified roots may contribute between 18 and 22% to the total subterranean biomass (Boddy and Watkinson 1995). Thus, approximately 2 ton of plant biomass may be deposited annually per hectare of forest in temperate regions due to the accumulation of leaf litter, fallen branches and periodically, whole trees. Together with carbon reserves, deadwood litter fall is estimated to retain between 1.1–3.7 and 0.1–0.8 kg/(ha yr) of nitrogen and phosphorus, respectively, which serves to immobilize essential nutrients until decay is achieved (Boddy and Watkinson 1995; Dighton and Boddy 1989). Without decay, nutrient reserves would accumulate and eventually halt ecosystem productivity.

Saprotrophic wood-decay fungi play a pivotal role in the ecology of forests as they are the principal agents of wood and chitin decomposition and hence nutrient and energy fluxes (Dighton 1997). Unsurprisingly, fungi therefore represent the dominant microbial biomass of the forest floor and soils of many forest ecosystems, of which 60% may be due to decomposer basidiomycetes. Energy flow through woodland ecosystems is ultimately dependent on a limited mineral nutrient availability. Consequently, the balanced cycling of mineral nutrients within woodlands is central to ecosystem

functioning, and fungal participation in mineral cycling is extensive (Boddy 1991; Dighton and Boddy 1989; Wainwright 1992). Fungi have been shown to be capable of all major nitrogen transformations except nitrogen fixation, although certain fungi may sequester nitrogen from nitrogen fixing bacteria within wood and fungal sporocarps (Barron 1992). Fungi can utilize organic (during decay) and inorganic (solubilized by organic acids and siderophores) phosphorus sources and many fungi produce siderophores to sequester limiting supplies of bioavailable iron (Cromack and Caldwell 1992). Mineral resources are transformed into new fungal biomass, organic acids, and humic substances. Indeed significant quantities of nutrients may be immobilized as fungal biomass, and this immobilization has resulted in the adoption of management practices such as burning to eliminate woody debris from forestry sites prior to re-plantation, thereby reducing potential competition for mineral nutrients between fungal biomass and tree transplants (Dighton 1995). Nutrients sequestered as fungal biomass may be released subsequently as a result of processes such as interactions with other fungal individuals or bacteria, by physical damage or invertebrate grazing and withstanding these, by senescence and death of the mycelium (Boddy and Watkinson 1995).

The biological diversity contained within woodland ecosystems may be exploited for practical and aesthetic gain. Traditional methods of exploitation have involved collection or cultivation of fungi for food, e.g., truffle fungi, *Lentinula edodes* (Shii-take), and *Pleurotus ostreatus* (oyster mushroom) as well as many other edible woodland fungi. Wood colonized by certain fungal species may be employed to generate valuable timber products. For example, "Brown oak" veneer timber (*pourriture rouge dur*) is produced by *Fistulina hepatica* colonizing heartwood, *Chlorosplenium aeruginascens* is used in the commercial production of Tunbridge ware, and wood containing interaction zone lines is turned to produce decorative artifacts. Novel methods of exploitation may involve the application of fungal decay systems to convert a range of renewable lignocellulosics into protein, fermentable sugars, and other products, or to bioremediate certain recalcitrant pollutants. Appreciation and understanding of fungal activities, relationships, and distributions in natural ecological contexts may provide insight into methods of searching and screening for useful products and conversion systems, and support the management and conservation of our valuable global resource. As Hawksworth and Colwell (1992) succinctly expressed as part of the Microbial Diversity 21 statement, saprophytic fungi, in common with other microbes, "are vital to the function of the Earth's ecosystems and biosphere. As major contributors in biogeochemical cycles, they perform unique and indispensable activities in the circulation of matter in the world, on which all larger organisms, including humans, depend. They constitute a genetic resource of great potential for contributing to the sustainable development of the planet as well as human, animal, and plant health. Urgent attention is required

to redress our ignorance on many key aspects of their scientific understanding, their distribution and functions."

3 THE FUNGAL HABIT

Filamentous fungi are immobile but may achieve motility by virtue of apical growth of hyphae from a supporting substratum. The indeterminate and versatile development of fungal mycelia is directed by interactions with the dynamic abiotic and biotic environments, the latter involving both inter- and intra- specific interactions between fungi and between fungi and other organisms. As heterotrophs fungi are nutritional opportunists, having evolved an array of optionally programmed developmental pathways to take advantage of changing local circumstances and resource fluxes (Rayner 1996). Fungi often possess a more restricted degradative repertoire compared with many bacteria, but fungal mycelia possess clear advantages over unicellular or short-chained growth morphs for invading and degrading solid substrata. Fungal mycelia absorb nutrients through a high contact surface area and may penetrate substrata by generation of mechanical pressure and extracellular degradative enzymatic and chemical activity. Mycelia are capable of nutrient translocation to a greater or lesser extent, and therefore have the capacity to support exploration from one nutrient rich source to another, thereby transcending regions of nutrient deprivation.

Highly specialized multihyphal structures such as rhizomorphs and mycelial cords are produced by a variety of wood decay, root pathogenic, and ectomycorrhizal fungi, such as *Armillaria* spp., *Phanerochaete velutina*, *Hypholoma fasciculare*, *Phallus impudicus*, *Tricholomopsis platyphylla*, and ectomycorrhizal *Leccinum scabrum* (see Boddy 1992; 1999; Rayner and Boddy 1988 for review). These linear organs are often evident at the interface of surface litter and soil in boreal, deciduous and tropical woodlands, where they may contribute to stabilizing woodland litter against weather erosion particularly on steep slopes (Lodge and Asbury 1988). Some may even form networks within the tree canopies of tropical forests (Hedger et al. 1993). Cords are organs of migration and exploration, with more efficient rates of biomass deployment and translocation of water and nutrients, compared to a more diffuse hyphal network, allowing the individual to transcend vast distances of often hostile regions or inhospitable nutrient deserts. Cords also provide resistance to the damaging effects produced by the soil microbial and microfaunal communities. The voracious opportunistic scavenging and persistent biomass involved may also facilitate nutrient storage. Cord formers are commonly particularly combative and are capable of producing rapid decay in nature, thereby making significant contributions to nutrient cycling (Boddy 1999; Boddy and Watkinson 1995; Chapela et al. 1988; Coates and Rayner 1985a).

Cord formation may be promoted by resource depletion (low C:N ratios and nutrients such as copper and calcium), recognition or contact with an antagonist, and reduced water

potential (Rayner and Webber 1984; Watkinson 1999). Directional growth of cords towards resource baits may possibly be mediated by volatiles or soluble diffusates, and involves coordinated redeployment of mycelial biomass following detection of localized resource base. Different species display different foraging strategies, subject to resource quality, quantity and distribution, biotic factors and microclimate (e.g., Boddy 1999; Donnelly and Boddy 1998; Wells et al. 1999; 2001; Zakaria and Boddy 2002). These linear organs can interconnect heterogeneously dispersed nutrient sources to form coordinated resource distribution networks (Boddy 1999), and may translocate different nutrients independently even bi-directionally, e.g., certain ectomycorrhizal cords may translocate carbon from tree roots to the growing mycelium, and mineral nutrients and water in the opposite direction.

4 FUNGAL ASSOCIATIONS AND DECAY

Fungal communities occupying forest ecosystems are dynamic and usually diverse, consisting of assemblages of organisms that interact with their surrounding abiotic and biotic environment (Boddy 1992; Rayner and Boddy 1988; Shearer 1995; Widden 1997; Zak and Rabatin 1997). Nevertheless, the unit community for each resource type often reveals distinct dominants and recurring associations of species during degradation (Dighton 1997; Frankland 1992). Consequently, prominence has often been given to the sophistication of the nutritional physiology of individuals when attempting to understand or explain fungal community development and successions. Thus, many explanations have centered around leagues of simple substrate groupings, or assemblages of individuals with particular degradative repertoires, simply following changes in resource quality as decay proceeds (e.g., Park 1968). Such a simplistic view should be considered with caution, as it tends to obscure other important ecological adaptations. It is also suggested that the term succession itself should be used with caution when describing fungal community development, particularly within certain resources such as wood. Terms such as substratum succession (Park 1968), and resource succession (Frankland 1992) may be misleading in suggesting simple order, thereby obscuring the actual complex multi-dimensional dynamics involved, which may follow a diverse array of optional pathways (Boddy 1992; Cooke and Rayner 1984; Renvall 1995). However, Frankland (1992) emphasized that the term succession should “accommodate a multi-dimensional phenomenon not a simple linear process” so as to include features such as environmental fluctuation, cycles and disturbance, life-cycle changes, and mycoparasitism without replacement.

But how should fungal ecology be best studied? Synecological studies are valuable in investigating the range and complexities of fungal communities, particularly when underpinned with appropriate sampling techniques and statistical analysis. Considered autecological approaches can

then target specific hypotheses. However, the indeterminate nature of the fungal mycelium clouds palpable identification of an appropriate scale of study, as interactions occur within a continuous hierarchy of spatial and temporal scales (see “Modeling Fungal Decay Communities”). Many patterns identified at a small scale may become less evident or significant at larger scales and vice versa. Furthermore, such features may be relatively insignificant under a particular set of environmental conditions, but ecological change or disturbance may augment their importance. Thus information derived at a variety of temporal and spatial scales is clearly most likely to contribute to a genuine understanding of fungal ecology. The future challenge will be linking information derived from differently scaled studies in an appropriate manner.

5 DESCRIBING THE ROLES OF FUNGI

Understanding the pattern of occurrence of an organism or population is largely a problem of identifying its realized niche. This task involves the identification of not only the space–time occupied by the organism in nature but also the role within that space–time during interaction with the biotic environment (Cooke and Rayner 1984). In turn, this leads to the problem of how best to classify the role of an organism. Apropos, appreciating the ecological (Andrews 1992; Pugh and Boddy 1988) and nutritional strategies displayed by individual fungi is a useful first consideration. Here I will consider the latter only.

An individual filamentous fungus may achieve an absorptive heterotrophic life-style via biotrophy or necrotrophy, either exclusively or sequentially. Fungal residents of woodland ecosystems exhibiting biotrophy include the (neutralistic to mutualistic to parasitic) endophytes and (mutualistic to parasitic) mycorrhizae, whereas archetypal necrotrophic representatives include certain (parasitic) pathogens and the decay saprotrophs of dead wood- and litter-tissues. These broad activity categories represent convenient but sometimes restrictive descriptors for the ecologist because they may obscure the dynamic nature of the relationship. In the next section I wish to avoid discussion on the boundaries of classification schemes, but will consider the wood-decay activities displayed by the associations mentioned above, so as to illustrate the spatio-temporal complexity and versatility of filamentous fungi. We as mycologists often focus on the wood-decay activities of true wood and litter saprotrophs, sometimes at the expense of recognizing the prevalence of such activities to other association habits. These association categories merely represent distinct points within a continuum of overlapping activity groups. Therefore, certain individuals may be classified simply as wood or litter inhabitants or as mycorrhizae, but others may decay both wood and litter, or may be mycorrhizal but also cause lignocellulose decay. Moreover, the nature of interactions between individuals may alter with life-cycle stage or environmental circumstances. An

individual may thus commence as an endophyte, then develop as a latent pathogen, and subsequently adopt a more saprotrophic role, following senescence of the host. The specificity of relationships also varies. Consequently, some fungi will be restricted to a particular biome, geographic area, specific resource, host species, part or cell-type within a host species. Others may be nonselective, ubiquitous generalists, such as the opportunistic decomposers. Furthermore, mycelial patterns may operate over a range of scales, from an individual pine needle (Kendrick and Burges 1962), to an entire forest (Smith 1999) or even at a global scale (Arnolds 1997).

Finally, the heart rot and active pathogenic activities of fungi causing death and decay of standing trees was described in the first edition of this text and will therefore not be considered here (Boddy 1991). The structural, chemical, and microclimatic features prevalent in living wood and the biology, ecology, and epidemiology of fungi causing its decay have been reviewed elsewhere (Butin 1995; Rayner and Boddy 1988; Schwarze et al. 2000; Smith et al. 1992; 1999; Tainter and Baker 1996; Vasilias 2001; Woodward et al. 1998).

5.1 Endophytes

Endophytes are defined as organisms that grow within a living host plant but not strictly within living cells, thereby deriving nutrition without detrimental symptoms for the host. It is believed that the living stems, twigs, and leaves of most woody plants contain symptomless endophytes, but because of their clandestine nature their prevalence is ambiguous, and identification of their presence relies on arduous techniques such as careful isolation from surface sterilized tissues and histological studies (Petrini 1991). Numerous endophytic species may be potentially allied with an individual host (e.g., 85 different fungal taxa have been isolated from root and stem tissues of *Alnus*). Moreover, host specificity varies, with some endophytes displaying little host specificity (e.g., *Phomopsis* has been isolated from *Buxus*, *Ilex*, *Hedera*, *Ruscus*, *Ulex*, *Pinus*, *Fagus*, *Juniperus*, *Fraxinus*, and *Quercus*; and anamorphs of *Xylaria* isolated from *Fagus*, *Fraxinus*, and *Quercus*) whereas others, such as *Peniophora* and *Vuilleminia comedens* tend to be more stringent (Griffith and Boddy 1990; Fisher and Petrini 1990). Although resident endophytes may not produce disease symptoms as such, their occupation is not without effect on the host.

The leaves or needles of many if not most plants contain fungal endophytes, estimated at a supporting cost of up to 0.5% of annual needle production in the case of conifer needle endophytes (Carroll 1992). However, the presence of some endophytes, such as *Phomopsis oblonga* in elm stems (Carroll 1991), may confer a selective advantage on the plant host, either due to secondary metabolite production or conceivably due to competitive exclusion, thereby protecting against pathogen invasion particularly insect attack or larvae development. Some may even be potential pathogens of

other competitor plants, with obvious consequences for the plant community, and have therefore been investigated as a potential biological control system (Leuchtman and Clay 1988).

Endophytes may also be involved with initiation of decay following stress or death of host tree tissues. Many endophytes, such as the Xylariaceae and their anamorphs, *Daldinia* and *Hypoxylon* spp. and *Nodulisporium*, are known wood-decay species. Some are observed to fruit extensively on very recently dead fallen logs, or form extensive primary decay columns or strip cankers (e.g., by *Eutypa spinosa* in European beech) very rapidly following host tree stress, particularly after drought (Hendry et al. 1998). Observation of such rapid and massive decay column formation has identified the role of endophytic morphs or latent invaders, distributed as somatically compatible or clonal latent propagules, dispersed within functional sapwood of standing trees. Such propagules may then be rapidly activated due to host stress such as declining wood water content and the growing spatial domains of individual genets may then converge and merge, thereby securing early and spatially extensive infection of dead wood (Boddy 1992; 1994). Latent presence of propagules in living bark tissues allows rapid and extensive invasion of dead attached twigs or recently felled logs (Griffith and Boddy 1990). However, detection of propagules does not necessarily implicate that species in initiating decay, as some endophytes have been isolated from host species known infrequently to support decay columns of these latent colonizers (Boddy 1994; Petrini 1991). This seemingly destructive activity within stressed and functionally redundant tissues may also be of ecological advantage to the tree, resulting in natural pruning of expensive nutrient sinks and the subsequent mobilization of nutrient resource.

5.2 Mycorrhizae

Mycorrhizae are mutually beneficial associations formed between the majority of plant and tree species and certain biotrophic soil fungi. The evolution of such a symbiosis provides a means through which the mycobiont can acquire most of its carbon from the photobiont mainly in the form of hexose, whilst donating mineral nutrients, particularly phosphorus and nitrogen, and supplementing plant water requirements. Plant resource acquisition and uptake is enhanced by the presence of a massive mycobiont hyphal surface-area, the activity of which improves mobile resource uptake and is usually solely responsible for uptake of most immobile resource. Affinity for and uptake of nitrogen as ammonia and phosphorus as phosphate is greater for the mycobiont than for the uninfected root. Fungi as mycorrhizal associates may protect against soil pathogens and environmental stress and contribute to the survival of most plants within the natural woodland environment (Smith and Reid 1997).

Some mycorrhizal fungi show little host specificity; e.g., *Amanita muscaria* may associate with conifers such as

Pinus and *Picea*, and with deciduous species such as *Betula*. Such nebulous specificity may allow formation of connecting networks, linking individual plants of the same and different species, in source–sink relationships via communal mycorrhizal fungi. Such conducting and communicating networks may be most significant during for e.g., the translocation of nutrients by a more mature plant community, to support the adjacent growth of stressed or shaded seedling plants (Trappe and Luoma 1992). The ecological significance of such resource sharing in sustaining primary production in natural forests, heathlands, and grasslands is currently the focus of research attention (e.g., Simard et al. 1997). Other mycorrhizal fungi exhibit more strict host specificity; for e.g., *Alpova diplophloeus* will only associate with *Alnus* spp. Nevertheless, the development of general successions of mycorrhizal fungi are often recognized during forest maturation, the individual occupants of which have been associated with *r*- and *s*-selected strategies and the nature of the litter resource. The ecological strategies of individual mycorrhizae have important implications for forestry practices with respect to choice of mycorrhiza inoculum for plantation within woodlands at different stages of development.

Four broad types exist; the vesicular-arbuscular- (VAM) or arbuscular- (AMF), orchid-, ericoid-, and ecto- mycorrhizae. The VAM or AMF generally occupy environments where phosphorus is the main growth limiting nutrient. They dominate forests in tropical climates, but some temperate trees such as sycamore, ash, and poplars have AM, and some such as willow can form both AM and ectomycorrhizal associations. Ectomycorrhizae, however, are supreme in forests on moder, mull, or brown earth soils in temperate regions of moderate latitude and altitude (e.g., Read 1991), and in relatively infertile soils particularly where nitrogen and phosphorus uptake is curtailed. The ectomycorrhizal fungal mycelium sheaths root cortical cells, forming a Hartig net, and extends through the litter and surface soil layers forming a foraging, ramifying network. The substantial mycelial investment involved implies that prolonged associations tend to occur. The biology, ecology, and biotechnological application of mycorrhizae have been reviewed and described in many excellent texts, for e.g., Allen and Allen (1992); Carroll (1992); Smith and Reid (1997); Podila and Douds (2000). This section will focus only on the saprophytic activity of mycorrhizae in temperate forest soils.

Conventional belief has been that the main function of mycorrhizal fungi is uptake and translocation of mainly immobile mineral nutrients released by free-living saprotrophs and that saprotrophy is generally lacking in mycorrhizal fungi. Indeed, nutrient flow between plant roots, ectomycorrhizae, and interacting saprotrophic non-mycorrhizal species has been demonstrated. For example, the wood-decay species *Hypholoma fasciculare* evidently lost phosphorus to mycorrhizal *Suillus variegatus* (Lindahl et al. 1999), whereas, carbon donation from *Pinus sylvestris* seedlings to mycorrhizal partner *Suillus bovinus* was visualized and declined in the presence of the decomposer

species *Phanerochaete velutina*. This latter species subsequently accumulated the labeled carbon source (Figure 1; Leake et al. 2001). Other examples of competition between mycorrhizae and saprotrophs have been reported (Shaw et al. 1995). However, the extreme mycorrhizal dependency exhibited by achlorophyllous plants is testimony to the role of fungal associates in translocating carbon to host plants (Leake 1994). The significance of similar carbon donation by

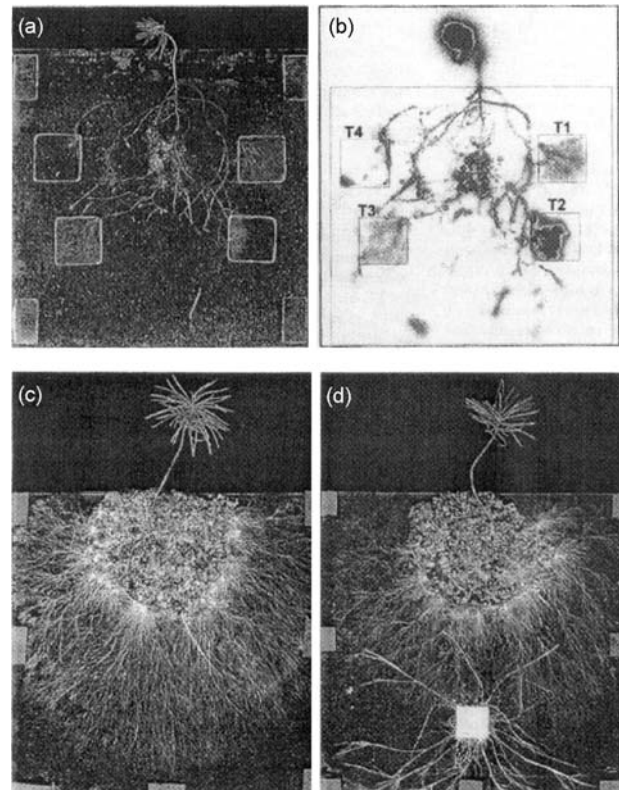


Figure 1 (a) Localized proliferation of mycelium of *Paxillus involutus* in mycorrhizal association with *Pinus sylvestris* on entering discrete patches of nonsterile forest litter in trays placed in an otherwise homogeneous peat substrate. (b) A digital autoradiograph of the microcosm shown in Figure a, 5 days later. The autoradiograph was acquired 48 h after ^{14}C pulse labeling of the shoot of the *P. sylvestris* seedling. Over 50% of the ^{14}C detected in the mycorrhizal mycelium (excluding roots) was allocated in the recently colonized litter patch labeled T2. (c) A control 20 × 20 cm microcosm with mycelium *Suillus bovinus* mycorrhizal with *P. sylvestris*, forming an almost complete covering of the surface of the peat. (d) Similar microcosm in which the mycelium of *S. bovinus* met and interacted with the mycelial cords of the wood-decomposer fungus *Phanerochaete velutina* growing from a wood block. Note the difference in density of cover and extent of mycorrhizal mycelium compared with (c). From Leake et al. (2001); Reproduced in black and white from color images.

mycobionts to autotrophic plants is now being investigated. Thus, it is becoming increasingly evident that, particularly in more acidic woodland environments, mycorrhizal fungi influence the degradation of leaf litter and woody debris. For example, mycorrhizal activity is stimulated by addition of litter to microcosms, and is maximal in temperate and boreal forests when nutrient litter supply peaks (Leake and Reid 1997; Unestam 1991). Furthermore, visual evidence of penetration of litter material by ectomycorrhizal hyphae (Leake et al. 2001; Figure 1; Ponge 1990), and nutrient release measurements following ectomycorrhizal (*Suillus bovinus* or *Thelephora terrestris* with *Pinus sylvestris*) colonization of litter patches (Bending and Reid 1995), all implicate a saprotrophic role for the mycobiont. Ericoid- and some ectomycorrhizal fungi, commonly found dominating ecosystems where nitrogen and phosphorus reserves often reside within accumulated organic matter, are known to produce extracellular enzymes capable of decaying complex carbon polymers present in litter and soil, thereby exposing and mobilizing otherwise trapped mineral nutrient reserves (Leake and Reid 1997). Proteins, amino acids, chitin, nucleic acids, phospholipids, and sugar phosphates all serve as nitrogen- and phosphorus-rich organic materials. Enzyme capacities so far identified include proteinase, peptidase, chitinase, acid and alkaline phosphatase, phytase, DNAase and RNAase, polygalacturonase, cellulase, xylanase, tyrosinase, peroxidase, polyphenoloxidase (including laccase), and ligninolytic activity (see Leake and Reid 1997). The hydrolytic capability of different individual ectomycorrhizal fungi is diverse (Hutchinson 1990), some being capable of degrading holocellulose, lignin, and lignocellulose (Trojanowski et al. 1984), while others utilize protein for carbon and nitrogen and translocate derivatives to host plants (Abuzinadah et al. 1986). The latter capabilities are of primary importance where successful mycorrhizal associations are established within ecosystems where the carbon may be supplied to the mycobiont often entirely by the plant symbiont, but available nitrogen and phosphorus supplies are growth limiting to the fungus and then ultimately to the plant host.

It has been estimated that maintenance of the mycorrhizal association costs the host between 9 and 28% of the total photosynthetic production annually (Finlay and Söderström 1992; Leake et al. 2001; Vogt et al. 1992). The benefits of such a costly relationship to the photobiont are not only limited to nutrient scavenging and mobilization, and to the saprotrophic release of otherwise unavailable resource, but extend to the ability of the mycobiont to construct extensive and persistent mycelial networks. This allows retention of a mineral budget within a fluctuating and discontinuous nutritional environment, as the mycorrhizal rootlets undergo dynamic degradation and formation, thereby preventing leaching of important nutrient reserves to the surrounding rhizosphere. Leake and Reid (1997) discuss the ecological significance of degradative activities of mycorrhizal and nonmycorrhizal fungi. They argue that the massive mycelial biomass and localized degradative activities produced within

woodlands by mycorrhizal species, together with donation of carbon supply by host plant species, may render the degradative role of mycorrhizal fungi as significant compared to nonmycorrhizal saprotrophs, at an ecosystem scale.

5.3 Saprotrophs of Attached and Fallen Wood and Litter

Saprotrophic fungi are the principle decomposers of nonliving plant and animal detritus in the natural environment, thus recycling chemical elements back to the environment in a form other organisms may utilize. Filamentous fungi usually dominate wood and litter decomposing communities, but under particular ecological circumstances, for e.g., for wood in tropical ecosystems termites may predominate, and under waterlogged conditions bacteria may prevail (Rayner and Boddy 1988). Other wood and litter residents may include yeasts, bacteria, Myxomycetes and invertebrates such as Insecta, Oligochaeta, Acaria, and Nematoda. These may influence fungal community dynamics and consequently affect overall decay rates, either via direct interaction, such as antibiosis or grazing of fungal mycelium or spores, or by indirect interaction through impact on the abiotic environment (Dighton 1997; Rayner and Boddy 1988). Thus, invertebrate activity can increase the exposed surface area for decay activity, may provide channels for mycelial invasion, and also improve aeration. Invertebrate waste and remains will also increase local available nitrogen supplies. Therefore, decay of litter and wood in forest systems are ecologically significant and complex processes, produced by intricate and dynamic communities.

5.3.1 Leaf-Litter Decomposition at the Substratum Community Scale

Estimates for litter production in temperate woodlands range from 3.8 ton/ha/yr for oak (*Quercus petraea*) and 5.7 ton/ha/yr for Norway spruce (*Picea abies*), although tropical forest litter production may be twice this. Leaves form the bulk of litter material, representing 65–75% of the total nonwoody input to forest ecosystems. The overall process of leaf decomposition involves the activities of fungi, bacteria, and animals, to produce a humic product, which becomes incorporated into the mineral soil fraction. Litter decomposition may take months (e.g., for *Ulmus* and *Fraxinus excelsior*) or years (e.g., for *Pinus sylvestris*, *Quercus*, *Fagus sylvatica*), and may accumulate to form layers if litter production is high and decomposition rates are low. Fungal successions on leaves as they mature, senesce, die and fall, are well characterized and described in detail elsewhere (Andrews and Hirano 1991; Frankland 1984; 1998; Dix and Webster 1995; Kinkel 1991; Ponge 1991). Unfortunately, investigations tend to focus on the generation of lists of species rather than enquiry into the underlying processes involved. Exceptions that attempt to explore the fundamental processes driving population and community dynamics

include application of "Life-history strategy" concepts (Andrews and Harris 1986), "Island theory" (Andrews et al. 1987; Wildman 1992) and a dynamic "Patch occupancy" model (Gourbière et al. 1999). Populations of fungi colonizing needles and leaves are governed by both external climatic effects with implicit immigration rates, and by fungal interaction effects such as competition and successional associations (Gourbière et al. 2001; Kinkel 1991). Colonization by phylloplane filamentous fungi and yeasts may commence immediately following emergence of a new leaf, and some species may already have a prior colonization history within the bud itself. Fungal spores deposited from the atmosphere must first survive stressful conditions, such as desiccation and rapid wetting, and intense UV and visible light as well as other environmental and climatic factors. Subsequently, primary colonizers overcome leaf inhibitory structures and compounds as well as microbial competition, whilst deriving nutrients via absorption of simple atmospheric nutrient supplies and leaf cell exudates.

Phylloplane and litter studies usually involve cultural methods: either plating of macerated portions or washings from leaf tissue, or of surface-sterilized portions (to distinguish between true colonists and nonviable propagules). However, Frankland (1998) cautioned that plate isolations might generate misinformation by merely selecting certain species from a complex of substratum successions. The isolation of certain species may better indicate the prevalence of dormant survival propagules, than their actual activity within the substratum. Direct observation following brief incubation in damp chambers is also useful, or following staining of leaf surface impressions produced in nail varnish or clear sticky tape, or even via electron microscopy. These different techniques have revealed that many incidental spores are in fact not viable, and that the pattern or succession of true colonizers is remarkably consistent for any particular leaf species, despite the diversity of propagules present. Early colonizers tend to be weak parasites or epiphytic saprophytes, e.g., certain yeasts and the cosmopolitan species *Aureobasidium pullulans* and *Cladosporium herbarium*, their activity often being restricted to the phylloplane until the onset of leaf senescence. The status of some species may indeed change from epiphyte to endophyte or even to weak parasite, depending on the interaction dynamic with the host plant defenses. Endophytes, pathogenic, parasitic or symptomless, may occupy up to 75% of leaves within a site. However, despite the diversity of endophytic genera involved, which may include *Phomopsis*, *Cryptosporiopsis*, and *Phoma*, the dominant mycoflora appear to be characteristic of the host species, regardless of host geographical distribution, thereby indicating a close evolutionary relationship between plant and fungus (Petrini 1991). Frankland (1998) also recognized the influence of host substrate on fungal species composition. Comparison of successions on different litter and nonwoody plant debris, revealed that the main differences in species composition were the early stage weak parasites, and that the duration of the decay succession, could be attributed to

resource quality, particularly nitrogen content and also to levels of fungi-inhibitory tannins. So, *Quercus* litter with the highest C:N ratio and tannin content was the slowest litter to decay, whereas *Fraxinus* with the lowest, produced the shortest decomposition period. In fact, during the decomposition of litter collected from a single source, but allowed to decay in adjacent but environmentally dissimilar habitats, similar successions were observed but with different temporal scales (Frankland 1998).

Senescent leaves together with resident mycoflora will eventually fall to the litter or soil surface. Some phylloplane fungi (*Aureobasidium* spp. and *Cladosporium* spp.) may persist, and some may even complete their (sexual) life cycle during this phase. However their consequential net decomposition may be very low. Litter is rapidly colonized by certain soil-inhabiting fungi (e.g., *Penicillium*, *Trichoderma*, and *Fusarium* spp.), which appear to cause little direct litter decomposition, but may produce significant indirect effects, such as synergistically increasing decay rates with litter-agarics. The early ruderal strategists are progressively replaced by saprotrophic communities, which decay leaf surface waxes, pectins, and the lignocellulose complex itself. Later significant decay stages are associated with the litter-basidiomycetes, especially agarics such as *Mycena*, *Marasmius*, and *Clitocybe*, which form a significant portion of fungal biomass within litter. Such species are capable of cellulose and hemicellulose hydrolysis and often also ligninolysis, as well as the detoxification of litter phenolics. *Mycena galopus* is often prevalent in temperate litter, displaying little resource specificity and causing typical white-rot, but is never found in bulky woody debris. Evidently, factors other than lignocellulose decay potential are significant in determining the part an individual may play in litter decomposition. For example, explanation of an apparent paradox co-existence of two litter saprotrophs *M. galopus* and *Marasmius androsaceus* both utilizing the same resource but possessing different colonizing vigor, involved microclimatic factors and another trophic level, i.e., preferential grazing by a mycophagous collembolan (Frankland 1998). Certain basidiomycetes show preferences for particular litter types, possibly due in part to the stimulatory effect that certain litter flavonoids have on their growth. So, for example, the growth of certain *Clavaria*, *Collybia*, *Marasmius*, and *Mycena* species is stimulated by addition of minute quantities of taxifolin glycoside to growth media. However, differences between representative communities occurring on angiosperm and coniferous litter, were attributed more to pH than to leaf phenolic properties (see Carlile et al. 2001 for references). Agarics are also most significant within deeper litter layers, where the improved moisture regime is conducive to basidiomycete growth and they are sheltered from fluctuating climatic conditions. The significant involvement of small soil animals in nutrient cycling, promoting litter decomposition via leaf fragmentation, nitrogen input, and detoxification of phenolics is reviewed elsewhere (Dix and Webster 1995; Ingham 1992).

5.3.2 Attached and Fallen Wood Decay at the Substratum Community Scale

Wood degradation is a relatively slow process that may in temperate woodlands, take tens of years for the decomposition of small branches to several hundred years for large trunks. The heterogeneous and polymeric nature of wood together with low nitrogen and phosphorus content limits decay of woody resources to certain fungi and less significantly to a few bacteria.

The 1980s and early 90s saw major advances in our understanding of the ecology of wood-decay fungi. This is comprehensively reviewed in several extensive and excellent texts (Boddy 1992; Rayner and Boddy 1988; Renvall 1995). Here I will simply present a brief outline. Investigations of indeterminate fungal growth within most natural environments present the ecologist with numerous constraints and practical challenges. However, the colonization of wood by fungi, particularly the higher fungi, provides the ecologist with an unparalleled opportunity to map the spatial activity domain of individuals *in situ*. Such systems can therefore provide a superb experimental system and conceptual framework for mainstream ecology. Competitive interactions dominate fungal communities within wood (Boddy 2000). Consequently, distinct territories prevail and are delineated within the wood by the production of antagonistic reactions such as pigmented zone lines, and colored activity domains indicated by different types of decay. Moreover, careful examination of wood sections may also reveal relic zone lines indicating the former occupants of woody domain. The community structures of colonized wood at various stages of decay have been mapped and the features affecting community development have been studied, using an astute but simple method based on these principles (Boddy 1992; Rayner and Boddy 1988). Essentially the technique involved sectioning wood, and recording the delineated boundaries of spatial domains occupied by individuals, prior to their isolation onto agar media. Isolates were identified and somatic compatibility of Basidiomycota and Ascomycota assessed using pairing techniques to map the presence of individuals. Interspecific pairing studies were used to assess antagonism and to rank species within a hierarchy of combative ability, to aid inference regarding the order of colonization and replacement (succession) of individuals. Thus, a 3D map may be constructed by aggregating information from serial wood sections. This basic method has also been complemented with other studies involving manipulation of drying regime, temperature, water potential, and gaseous environment. The approach has been used to study the community structure of attached branches and twigs of oak (*Quercus petraea* and *Q. robur*), Ash (*Fraxinus excelsior*), beech (*Fagus sylvatica*), and birch (*Betula pendula*) (Boddy 1992; Griffith and Boddy 1991a; Rayner and Boddy 1988). The majority of wood-decay studies have been concerned with basidiomycetes from early stages of decomposition, and with ascomycetes, moulds, and zygomycetes particularly from later decomposition stages

(Chapela 1989; Crane et al. 1996; Crawford et al. 1990; Lumley et al. 2000; Rayner and Boddy 1988). Host-wood species, physico-chemical properties, and microclimate govern the basic fungal community dynamics, as does the prior history of the substratum (Barron 1992; Butin and Kowalski 1986; Chapela et al. 1988; Griffith and Boddy 1990; Keizer and Arnolds 1990). Water distribution and its reciprocal relationship with aeration, were identified as principal determinants of colonization patterns (Rayner and Boddy 1988; Griffith and Boddy 1991b). Thus, the moisture relations of living trees inhibit fungal growth and decay by the majority of fungi, except for certain pathogenic and endophytic species. However, to overcome this, some fungi apparently employ a strategy of active wood desiccation to achieve appropriate conditions for establishment and decay (Hendry et al. 1998).

The decay process often commences in the standing tree, in attached lower or stressed branches (Rayner and Boddy 1988). Fungi may gain access either through wounds, tissues following microbial or stress damage or via lenticels or leaf scars. Studies have indicated that pioneer species such as *Stereum gausapatum*, *Phlebia rufa*, *Phellinus ferreus*, *Exidia glandulosa*, and *Vuilleminia comedens* in oak or *Daldinia concentrica*, *Hypoxylon rubiginosum*, and *Peniophora limitata* in ash, can colonize living or recently dead wood. The host tree may instigate a response to this invasion, by accelerating localized premature heartwood tissue formation, which contributes to restriction of the invading front. The identification of massive decay columns comprising a single individual extending for several meters along branches known to have been dead for a single growing season only, indicated the involvement of latent invaders (see "Endophytes") initially distributed within functional sapwood as "dormant" hyphal fragments or propagules. Mycelial growth and colonization would then activate from multiple inoculum sites, following stress alleviation for the fungus (usually drying sapwood), imposed by stress aggravation for the host. Ramets of an individual genet could then grow and unite by anastomoses thereby forming extensive decay columns apparently occupied by a single individual. Secondary invaders such as *Coriolus versicolor*, *Phlebia radiata*, *Stereum hirsutum*, and *Peniophora lycii* in oak, and *Radulomyces confluent* in ash, could invade and replace pioneers in already dead or decaying wood.

Decomposing dead wood gradually releases sources of nutrients (Harmon et al. 1994). Exposed stumps of felled trees, fallen branches and twigs, or cut timbers may become rapidly colonized by large numbers of individuals of fairly nonselective saprotrophs, thereby forming numerous smaller decay columns. Community structure and development is affected by the degree of exposure, contact with the ground or with other wood, and is influenced by microenvironmental conditions and the arrival mode of individuals. Exposed surfaces may be colonized by established air-borne spores of *Coriolus versicolor*, *Bjerkandera adusta*, *Stereum hirsutum*, *Chondrostereum purpureum* basidiomycetes or ascomycetes in the genus *Hypoxylon* or *Xylaria* commonly on hardwoods.

Such a mode of establishment often produces slower expansion of decay columns compared to that from ground contact, presumably due to the more stressful drying regimes. Buried or ground contact wood may be colonized by soil-derived spores, mycelia, or cords. Later decay stages may involve *Mycena galericulata* and *Pluteus cervinus* on hardwoods, or *Tricholomopsis rutilans* and *Paxillus atrotomentosus* on conifers. On very wet, well-decayed wood Dacrymycetales such as *Dacrymyces stillatus*, or discomycetes such as *Mollisia cinerea* may occur.

That different individuals produce different types and rates of decay is now evident (Worrall et al. 1997). Furthermore, the spatio-temporal combination of extracellular enzymes secreted by a fungus is dependent not only on its evolutionary heritage, but also on the local environmental and biotic conditions (Griffin 1994; White and Boddy 1992a,b). Few studies have related community structure and development with the decomposition process itself. This seems to be a little imprudent as both are obviously intricately linked. Notable exceptions (Coates and Rayner 1985a,b) have demonstrated that inoculation via large numbers of individuals produce slower net decay rates when compared with that for low spore loads, probably due to expression of alternative metabolic pathways related to antagonism between numerous small domains. However, several authors have observed enhanced decomposition rates by mixtures or sequences of fungi (Boddy et al. 1989; Deacon 1985).

5.3.3 Community Studies at the Forest Ecosystem Scale

Direct examination of twigs, branches, and logs for fruiting structures is a traditional method of surveying for basidiomycete and ascomycete activity. But the flora detected using this approach is often very different to that isolated by plating wood portions from interior wood regions. Inferences regarding community dynamics if based on sporophore surveys should be made with extreme caution as their appearance may bear little relationship to the arrival, activity or decline of the supporting mycelia and the relationship will vary for different individuals. Thus, the time taken for the mycelium to derive sufficient resource to devote to sporophore production may be one or more seasons but may be evident only temporarily and the active mycelium of some fungi may not produce sporophores until their final stages of colonization. Some, such as *Ganoderma* and *Fomes* produce perennial fruit bodies, which may survive for several years, whereas others may produce seasonal and/or ephemeral fruit bodies. For example, soft-rot fungi such as *Chaetomium* spp. are prevalent colonizers frequently only detected via plating techniques. Similarly, the dominant ectomycorrhizal species *Tylospora fibrillosa* in a Sikta spruce plantation was not detected in sporophore surveys, and species indicating most abundant fruiting represented only a very small abundance in association with sampled roots (Taylor and Alexander 1991). Therefore the phenology (climate and time of appearance) and abundance of fruit-bodies for different species must be

considered when interpreting survey data. However, careful experimental design and statistical treatment of such data can provide the ecologist with a useful approach to investigating factors influencing fungal community dynamics and decay at the forest ecosystem scale.

Microclimate, substrate quality determined by host species and decay stage, and forest history are cited as being the most significant determinants of fungal community structure in decaying wood (Lindblad 1998; Lumley et al. 2001; Rayner and Boddy 1988; Sippola and Renvall 1999; Vogt et al. 1992; Zhou and Hyde 2001). Other factors affecting species compositions include soil chemical properties (Ruhling and Tyler 1990), and vegetation type (Wasterlund and Ingelög 1981). Furthermore, the initial heterotroph community (bark beetles, ambrosia beetles, moulds, or decay fungi) has been found to influence decomposition or carbon flux in freshly cut Douglas fir (Progar et al. 2000). Multivariate analysis of survey data, such as detrended correspondence analysis (DCA), can indicate the relative importance of these variables for community structure and development within forest ecosystems. In dead-wood, decay state and microclimatic stress have been identified as most influential with some impact of soil conditions (Heilmann-Clausen 2001). Cluster analysis and ordination of microfungus communities in white spruce (*Picea glauca*) and trembling aspen (*Populus tremuloides*) fallen logs in disturbed and undisturbed boreal woodland sites revealed tree species to be most influential, followed by stage of decomposition and moisture content (Lumley et al. 2001). From the foregoing, the importance of appropriate moisture regime for decay community development is evident, indeed particularly wet forests have revealed relatively depressed respiration and decomposition rates (Progar et al. 2000). Community analysis can also suggest general trends relating to species diversity and community development. Communities develop relatively quickly and predictably at early decay stages, but become slower and more diversified at later stages as the impact of fluctuating microclimatic stress become more significant due to increasing wood porosity (Heilmann-Clausen 2001). Biodiversity is greatest in undisturbed deadwood, and tends to increase as decay proceeds (Lumley et al. 2001; Norden and Paltto 2001; Renvall 1995). Moreover, some late-stage decay species may be prone to local extinction due to strict successional associations formed between certain species (Niemelä et al. 1995).

The DCA of sporophore data from decomposing conifer trunks in northern Finland indicated the development of regular successions of wood-decay fungi. Differences observed among successions were due to associations with the prevailing microclimate and dependent on the resource capture strategies and combative ability of individuals (Renvall 1995). In short, environmental stress created succession pathways for specific saprotrophic groups. Such an inference is very much in accordance with the concept presented by Cooke and Rayner (1984), that community development pathways are initiated under varying degrees of high abiotic stress and/or low stress conditions following

disturbance and is subsequently directed by four influences; disturbance, stress aggravation, stress alleviation, and intensification of combat (Boddy 1992).

6 FUNGAL DIVERSITY, ENVIRONMENTAL CHANGE, AND CONSERVATION

Biodiversity is generally considered to be fundamental to ecosystem functioning and stability. Thus, if this tenet is extrapolated to fungi in forest ecosystems, fungal biodiversity is critical to global geochemical cycles. A well-cited global estimate for fungal biodiversity is in the range of 1.5-M species (Hawksworth 1991; 2001). However, at a local level, appropriate monitoring of fungal diversity can identify ecological and climatic trends, provide a database for spatio-temporally predictive biodiversity models, and inform appropriate conservation strategies at both the regional and global scale (Arnolds 1997).

Most local diversity studies are based on survey of conspicuous fruit bodies of higher fungi, either represented as presence, number, or biomass within a plot. Diversity studies of ephemeral and sometimes sensitive fruit bodies will only represent a partial account of the resident fungi. Nevertheless, such studies are often valid because of their ease of (usually nondestructive) analysis over extensive sampling domains. Fruit body surveys are further justified because of the ecological significance of spore dissemination for the establishment of new genets, and due to the involvement of sporocarps in soil mineralization processes owing to developmental regulation of extracellular enzyme activities (Ohga et al. 1999). Moreover, the fruit bodies of many species are a valuable edible resource. However, sporocarp inventories of species with varying resource allocation to reproduction, especially for comparison of taxonomically and ecologically different groups, are fallible as a bioindicator of diversity. Thus, ruderal strategists are generally likely to fruit more frequently and transiently than combative strategists. Moreover, sporocarp productivity of different species are known to vary spatially (clustered or dispersed and associated with distributed substrata), temporally (yearly, seasonally or weekly), can mature at differing rates and persist for varying time periods (Egli et al. 1997; Vogt et al. 1992). Fruiting may also be influenced by environmental factors such as temperature and precipitation, natural and artificial amendments to soil and by local or nonlocal ecological disturbance (Straatsma et al. 2001; Watling 1995). In the case of certain mycorrhizal fungi fruit body development is affected by host genotype, age, provenance, edaphic factors, climatic factors, and supporting photosynthetic activity (Dix and Webster 1995). The relationship between individual visible fruiting structures and mycelium activity within substrata is therefore varied and complex, and considerable disparity has been identified between the two in numerous studies (Cotter and Bills 1985; Gardes and Bruns 1996; Yamada and Katsuya 2001). Notably, only about half of the ectomycorrhizal

community has been indicated by production of conspicuous epigeous fruit bodies (Dahlberg et al. 1997; Peter et al. 2001).

Sampling plot size, frequency, and distribution are also likely to influence the data set, as different ecological groups will operate over a range of spatio-temporal scales (Newton and Haigh 1998; Schmit et al. 1999). Indeed, it has been suggested that different ecological guilds would require differently sized and spatio-temporally distributed sampling protocols (Villeneuve et al. 1991). To compound such problems, Tofts and Orton (1998) declared that 21 years of recording species accumulation was insufficient to reliably estimate (extrapolate) the fungal biodiversity of the site, as the species accumulation-time curve had not yet started to plateau. At a practical level, the quality of a data set is even dependent on the expertise of the collector (Straatsma et al. 2001). However, sporophore surveys may be justified as a suitable method for warning of species disappearance due to detrimental external factors within a site, as evidence suggests that fruiting may be more sensitive to environmental stress than survival of the supporting mycelium itself (Termorshuizen and Schaffers 1991).

Despite these limitations evidence accrues, particularly from Europe and more recently the United States, suggesting that fungal biodiversity is in decline. The likely causes of decline are due to habitat loss and/or pollution. Harvesting of wild edible mushrooms is believed to have little detrimental effect on fungi, except where collection has involved damaging or exhausting the mycelium, or trampling or raking the soil (Arnolds 1995). Nevertheless, the environmental impact of large-scale commercial harvesting remains a contentious issue. Fungal habitat may be lost, with implicit reduction of fungal species diversity, either by deforestation, or because of commercial forestry management practices, such as the conversion to less-mixed or monoculture plantations, stand felling of a particular age, and the removal of coarse woody debris (Fridman and Walheim 2000; Høiland and Bendiksen 1996; Lindblad 1998; Norden and Paltto 2001; Ohlson et al. 1997; Straatsma et al. 2001). Red-List (endangered, vulnerable, or rare) species may be particularly sensitive, often showing a preference for large diameter logs in late decay stages, of which managed forests are largely deficient (e.g., Humphrey et al. 2000; Kruys et al. 1999).

Pollutants such as lead, sulfur dioxide, ozone, and oxides of nitrogen (direct or as acid precipitation) are known to affect biodiversity (Wainwright and Gadd 1997). Ectomycorrhizal communities are particularly threatened by pollutants such as sulfur dioxide and NH_x , probably due to forest soil acidification and nitrogen input, thereby rendering any potential mycorrhizal association less advantageous to the host plant community (Arnolds 1995). Elevated sulfur dioxide levels are known also to affect the phylloplane community, although some pigmented species may be less susceptible, thereby favoring their population growth (Magan 1993). The resulting impact on community interactions may have implications in the dynamics between pathogens and saprotrophs and the consequent natural limitation of plant diseases, and even on the subsequent decay rates of litter and

hence nutrient cycling (Newsham et al. 1992). Similarly, high levels of radioactivity occurring around the Chernobyl site have produced altered fungal community structures (Zhdanova et al. 1994).

Considering the absolute and relative diversity, fundamental ecological importance, some host-specificity and -exclusivity, and established and potential biotechnological significance of fungi (Hawksworth and Colwell 1992; Oberwinkler 1992), their limited conservation seems unjustified and surprising. For example, only four fungal species are protected by UK law, comparing pitifully with over a hundred flowering plants (Marren 2001).

7 MODELING FUNGAL DECAY COMMUNITIES

From the foregoing it is evident that significant limitations exist in both field- and laboratory-based studies of wood-decay fungal communities. Moreover, much of the information reported is qualitative and at best semi-quantitative. A complete understanding of the natural environment based on observation alone is probably unattainable due to its complexity, the appreciation of which may be approached in several ways. Conventional methods often involve an empirical or field based approach to investigate the activities of individual decay fungi and their temporal relationship with the abiotic and biotic environment. These may be analyzed statistically or further complemented with laboratory experiments. More rarely, fungal ecological investigation may involve the development of conceptual and experimental models based on general ecological theory (Carroll and Wicklow 1992). The use of conceptual representations or models has been instrumental in expanding the boundaries of our knowledge and understanding of the natural environment. A model in its broadest sense is a partial, simplified version of a real entity or system. The main value of models is that they allow us to first represent and then make measurements and predictions that would be otherwise awkward. Theoretical or mathematical models based on experimentally observed characteristics of fungi are increasingly being used, for e.g., in formulating hypothesis about their population dynamics and epidemiology (which will not be considered here and the reader is directed to Worral 1999). A particular advantage of linked models is the interplay between quantitative experimental data and theoretical predictions. It is therefore possible to test and validate the theoretical model by predicting the result of, for e.g., changing an environmental factor in the experimental system and then observing whether the predicted result occurs.

The development of theoretical or mathematical models to describe any real ecological system will always be contentious, given the inevitable simplification or reduction of the system inherently required by such an approach. However, appropriate theoretical models should allow inferences to be made regarding the underlying mechanisms

or processes driving ecological systems. Thus, the biological processes that regulate and generate patterns of organisation in fungal communities may be elucidated through the understanding of the interactive and feedback control steps involved within a working theoretical model. So, as Moorhead and Reynolds (1992) reasoned, "the process of developing a model tests our understanding of the system, identifies areas of uncertainty and, importantly, provides a means of examining alternative conceptual frameworks." Furthermore, mycology may offer important systems to study and test the suitability of general ecological theories and laws usually developed for determinate organisms, for application to indeterminate biological behavior. Rare examples of this approach include consideration of the ecological strategies of individual fungi (Andrews and Harris 1986; Boddy 1992), and testing the relevance of Island theory, and the Species-area curve, to certain fungal communities (Andrews et al. 1987; Newton and Haigh 1998; Wildman 1987).

The majority of natural environments display both spatial and temporal heterogeneity or patchiness in terms of both abiotic and biotic factors, which may profoundly effect the functioning and development of fungal communities (Ritz and Crawford 1999). So models that address environmental heterogeneity are the most likely to produce realistic theory. Certain distinctive biological attributes displayed by the fungal form should also be incorporated into any reasonable theoretical model. Features such as indeterminacy, interconnectedness, variation, and versatility (genotypic and phenotypic plasticity), all contribute to varying degrees and at different scales, to the success of filamentous fungi within the natural heterogeneous environment. As indeterminate life-forms the growth of filamentous fungi is potentially unlimited, unlike that of determinate life forms such as unicells and animals, which possess genetically programmed limits in both space and time. However this statement may be an oversimplification of the fact, as the longevity of indeterminate individuals and species does vary. Ruderals, for e.g., may persist only briefly for a few weeks, whilst some such as *Ganoderma* and *Armillaria* are renowned to persist for years. Nevertheless, the indeterminacy displayed by eucarpic fungi facilitates success within a heterogeneous environment, as hyphae have the potential to temporally sustain or spatially extend great distances, often spanning inhospitable conditions in time or space to encounter new resource bases. Indeterminacy may generate versatile responses to environmental unpredictability in a way that determinate organisms can only engage with by way of evolutionary selection or social behavior.

Indeterminate and modular life forms show some analogies and have sometimes been treated as theoretically equivalent (Trinci 1978; Prosser 1994). However, modular organisms develop by repeated addition of the same organizational unit to a pre-existing one, whereas mycelia are nonadditive as they operate as dynamic flow systems with branching, anastomosing and radiating components communicating at different scales and to varying degrees (Rayner 1996). Therefore, the temptation to apply modular models,

which incorporate iterative steps at a single reference scale, to indeterminate systems, should be considered with caution. The challenge will be to develop modeling frameworks that can link biological processes operating over multiple scales and with varying constancy, to large-scale or ecosystem behavior. Developing and therefore understanding this complexity would make a major contribution to both fungal and general ecoevolutionary theories.

7.1 Modeling the Growth of Hyphae and Mycelia

Many mycological models have attempted to represent and explain the growth and development of hyphae and mycelia in relation to their abiotic environment. The reader is directed to Ritz and Crawford (1999) for a review of some of the experimental approaches and mathematical models developed to date, and so these will not be considered in any detail here. Most have focussed on the foraging and space-filling properties of mycelia developing on resources distributed discretely or as gradients. Reaction-diffusion models have been derived to reflect hyphal growth and branching (Regalado et al. 1996) and different colony morphologies (Davidson et al. 1996). More recently a simple stochastic model accommodating some biological processes (inhibition by toxic metabolic products and nutrient uptake) was able to reproduce a variety of fungal growth patterns observed on solidified media (Lopez and Jensen 2002). However, there is a paucity of theoretical models that accommodate environmental topography, translocational source–sink relationships, and exploitation vs. exploration growth morphs, either individually or in combination. In an attempt to address these issues, work is currently being undertaken to develop a

generic spatially explicit model adopting a process-based approach, in which the fungal individual is defined by a set of measurable traits that describe physiological processes such as nutrient uptake, redistribution and growth (Figure 2). Each individual may be described by a characteristic set of traits and therefore community diversity may be accommodated within the model. Being spatially explicit where biomass is located in cells on a discrete spatial grid, the model permits regions of mycelia to interact within a neighborhood and change according to the local environment and context. Thus, the ethos of the modeling framework is that any system can be described by a set of processes that can approximate the system. By linking these processes to parameters that may be measured experimentally (physiological traits), a mechanistic understanding of those parameters influencing the overall organisation of the system can be obtained.

7.2 Modeling Fungal Community Dynamics

Modeling approaches for fungal communities, have received far less attention, perhaps because this is a most ambitious project, summed-up by Frankland (1998) as “unravelling the unpredictable.” Indeed, the work of Halley et al. (1996) represents one of the few attempts to model interactions within a multispecies fungal decay community. In this work, a computer based simulation model known, as a “cellular automaton” was developed to predict the decomposition of wheat straw by four saprotrophic fungi. The model was based on real experimental data obtained by Robinson et al. (1994) during studies on resource capture by interacting fungal colonisers of straw. Although this model was capable of reproducing some of the behaviors exhibited during the

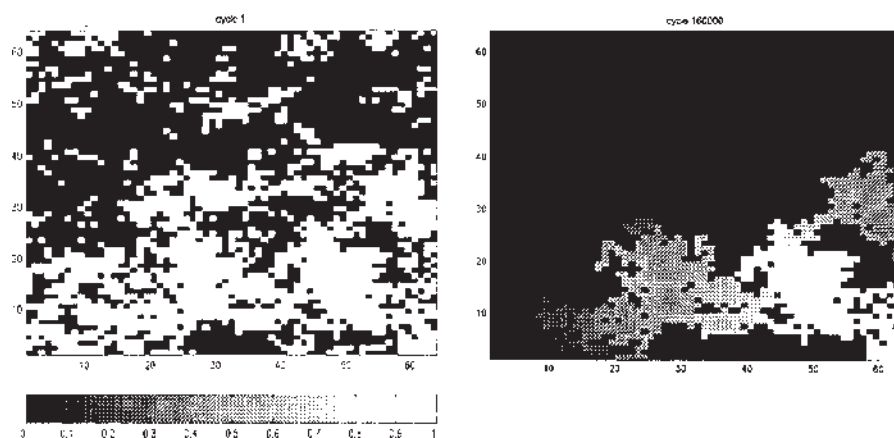


Figure 2 (a) Image showing the underlying soil pore space derived from real data using a CAT scan—with white to black scale representing pore to solid respectively. (b) Image showing the resulting fungal biomass gradient distribution obtained from the theoretical model with the mycelium growing from a single point and resource (bottom right hand corner) with white to black scale representing high to low biomass, respectively. The model is based on a set of biological processes, intended to characterize any fungal individual. By linking these processes to parameters that can be measured experimentally, a mechanistic understanding of which parameters are influencing the overall organization of the system can be obtained (Falconer R, Bown J, Crawford JW, and White NA, unpublished data).

experimental study, validation of the model was only possible on a qualitative basis. Furthermore, the model admittedly ignored important aspects of the fungal community such as sporulation and the coordinated behavior of the mycelium.

More recent modeling studies have attempted to examine and accommodate the complex and coordinated behavior exhibited by indeterminate mycelia. A stochastic cellular automaton for modeling the dynamics of two-species microcosm communities of differing patch size, revealed the significance of local and nonlocal interactions in generating the emergent behavior of mycelial systems (Bown et al. 1999). Importantly, the experimental system allowed for detailed quantitative spatial analysis of the community. This revealed that despite the predictability of the final interaction outcome irrespective of patch size, the finer-scale dynamics were highly dependent on non-local interactions (Bown et al. 1999; Sturrock et al. 2002). Furthermore, experimental studies indicate that fungi occupying large domains commonly display a higher combative success compared to those occupying smaller domains when challenged by the same species, and *in vitro* that younger mycelia may be less combative than more mature growth (Holmer and Stenlid 1993; Stahl and Christensen 1992). Consequently, despite the benefits of *in vitro* interaction studies in understanding the factors that influence the community dynamics of fungi, a major limitation exists in how such small-scale studies relate to the behavior at larger community scales. At larger scales it is probable that contrasting emergent behaviors may arise

because individuals in a group or patch behave differently to individuals that are isolated. Processes such as modification of the environment, resource translocation and hyphal networking or anastomosis (Rayner 1996) may all influence community development. Issues relating to scale have been a central problem in ecology over the last fifty years. Nevertheless, the importance of appreciating the relationships across different scales should be emphasized, as understanding and predicting large-scale ecosystem events, will have origins in and consequences for fine-scale phenomena (Levin 1992). Therefore, in the development of a complete understanding of fungal ecology it is necessary to address issues relating to scale and to adopt a hierarchical framework (Allen and Hoekstra 1992; Swift 1976).

In vitro mycelial interaction studies, particularly those evident on agar media, have often formed the basis of understanding or predicting *ex situ* fungal community dynamics. Furthermore, these have often centred on the pair-wise interaction between a limited number of individuals under experimentally defined environmental conditions (Boddy 2000). Data produced from combative interaction studies are often used to rank species in order of their combative ability, and hence to indicate sequences of fungal colonisation in the field. Correlation between antagonistic behavior in artificial culture and in the natural environment is mixed, and may be related to the spatial or temporal scale at which the data are collected or aggregated. Nevertheless, such studies represent a valuable approach to understanding fungal ecology, providing that any limitations are recognized,

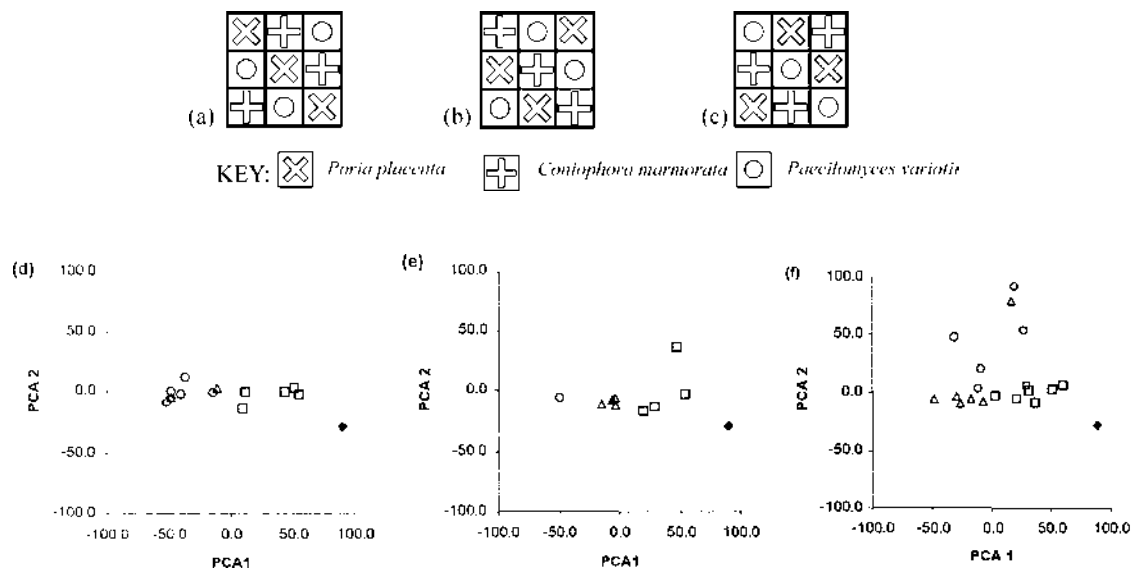


Figure 3 (a)–(c) Example maps showing the spatial distribution of fungal species in a 3×3 (equal proportion) tessellated agar tile interaction array at the onset of the experiment. Bold lines denote air-gaps between individual tiles. Dimension of each tile is 1 cm^2 . Symbols indicate the species inoculated onto each tile. (d)–(f) Plots of first and second principal components from analysis of interface classes and state transition classes of tessellated agar tile arrangements (a)–(c). $\diamond = 0$ week, $\square = 1$ week, $\triangle = 3$ weeks, $\circ = 5$ weeks incubation at 15°C . From Sturrock et al. (2002).

qualified and considered. According to general ecological terminology combative hierarchies may be either transitive (following a strict linear order of hierarchy) or intransitive (no strict linear hierarchical order) (Boddy 2000). Consequently, predicting the interaction outcome of more than two species may become extremely difficult, if not impossible, if the species under investigation display an intransitive hierarchy (White et al. 1998). Thus, the expansion from two-species interactions to three or more species may allow for the emergence of "higher-order interactions" involving greater complexity and even different biological phenomena, often resulting in shifts in interactive functioning (Culver 1992). Furthermore, the initial spatial configuration of three-species communities of equal inoculum and patch size, has been shown to impact on community development and reproducibility, and may contribute to the persistence of individuals which would otherwise be expected (based on binary pairings) to be eliminated from the system (Figure 3; Sturrock et al. 2002). In addition, fungal individuals can often display a variety of responses when subjected to ostensibly the same set of conditions (Figure 3; Halley et al. 1996; Rayner et al. 1995; White et al. 1998). Such stochasticity may be interpreted as offering the mycelium an ecological advantage within a dynamic and unpredictable heterogeneous environment. The complex stochastic and sensitive nature of community interactions is particularly evident within experimental community systems involving more than two species, which tend to demonstrate more heterogeneity among replicates with the same initial arrangement compared to binary-pairings (Sturrock et al. 2002; White et al. 1998). Agreement between agar based studies and those occurring in natural substrata is also likely to be influenced by environmental complexity (Boddy 2000). Water potential, gaseous regime, temperature, and resource quality have all been shown to significantly influence the outcome of interactions. Importantly, the sensitivity of the interaction outcome may not be solely associated with influence on colony extension rate, and that improved growth temperature regimes are not necessarily conducive to superior combative physiology (Sturrock et al. 2002).

From the above the limitations implicit within fungal models are clear. Frankland (1998) remarked that community development would become "predictable only when the mechanisms are understood and can be related to the multivariate situation." However, the contrary approach should be considered, explicitly that the process of developing a working model, will highlight the important underlying biological processes and mechanism driving community development, and identify new hypotheses for further investigations.

8 CONCLUSIONS

The roles of wood-decay fungal activities are therefore diverse and fundamental to forest ecosystem functioning. The fungal communities operating within the woodland resource

have been studied at a range of spatio-temporal scales. The future challenge will involve linking information derived at a range of scales in an appropriate manner. Theoretical modeling approaches have value here, the development of which can highlight the important biological features driving community dynamics and identify further hypotheses for testing. Such models may one day even have predictive value, an important asset in view of changing management practices, land use, climate, and other environmental pressures. However, future experimental studies and theoretical models should attempt to link fungal diversity, community structure, and dynamics to function, either in terms of nutrient cycling or biological control. The fungal ecologist is faced with challenging and exciting opportunities to study what is one of mankind's most valuable assets.

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The Biodegradation of Lignocellulose by White Rot Fungi

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1 INTRODUCTION

Lignocelluloses are defined as plant or wood cell walls in which celluloses and hemicelluloses are intimately associated with lignin. The role of lignin is to provide strength, serve as barrier against microbial attack, and act as water impermeable seal across the cell walls in the xylem tissue (Argyropoulos and Menachem 1997). Lignin is a phenylpropanoid polymer synthesized from the phenolic precursors, coniferyl, synapyl, and *p*-coumaryl alcohols (Sarkanen and Ludwig 1971). Free radical condensation of these precursors initiated by plant cell wall peroxidases results in the formation of a heterogeneous, amorphous, optically inactive, random, and highly branched polymer with at least 12 different types of linkages such as aryl-ether and carbon-carbon bonds connecting the aromatic nuclei. Such structural features impose unusual restrictions on its biodegradability (Hatakka 1994; Higuchi 1990; Kirk and Farrell 1987). Most biological macromolecules such as cellulose are largely linear polymers whose subunits are linked together by a repeating bond, and thus the mechanism of polymer degradation is generally centered around the common bonds. The complexity of the lignin polymer means, however, that it is not subject to enzymatic hydrolysis and the initial attack must be oxidative, nonspecific, nonhydrolytic, and extracellular (Hatakka 1994; Higuchi 1990; Kirk and Farrell 1987). Since lignin is particularly complex to biodegrade, the cellulose, hemicellulose, and other cell wall constituents with which it is intimately associated are

protected from degradation and as a result their bioavailability is very low. Lignin degradation, therefore, plays a central role in the earth's carbon cycle, since most renewable carbon is either in lignin or in cellulose and hemicellulose. This chapter will focus on the biodegradation of lignin by fungi, with particular emphasis on white rots, which are the most efficient degraders and the only known organisms that can completely breakdown lignin to carbon dioxide (CO₂) and water (H₂O) (Kirk and Farrell 1987).

2 BROWN ROT AND SOFT ROT FUNGI

Only a few organisms are capable of degrading the aromatic polymer lignin, the most efficient of which are fungi. Three groups of fungi are capable of lignin degradation (Eriksson et al. 1990): White rot, brown rot, and soft rot fungi (Table 1).

Brown rot fungi belonging to the basidiomycetes extensively degrade cell wall carbohydrates and only modify the lignin (Eriksson et al. 1990). Demethylation is the most obvious consequence of attack on lignin by these fungi. The brown rot fungi grows mainly in the cell lumen next to the secondary wall and cause a generalized, diffuse rot (Blanchette 2000). The residual wood is brown and often cracks into cubical pieces when dry. Brown rot fungi have an obvious preference for coniferous substrates (gymnosperms), which are softwoods. A survey of substrate relationships reported that 19% of North American basidiomycetes are brown rot fungi,

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Table 1 Lignin degrading fungi, their actions, and distribution

Organism	Subdivision	Examples	Actions ^a	Distribution
White rot fungi	Basidiomycetes	<i>Phanerochaete</i> sp., <i>Pleurotus</i> sp., <i>Bjerkandera</i> sp., <i>Trametes</i> sp., and <i>Phlebia</i> sp.	Mineralize lignin to CO ₂ and H ₂ O; some species preferentially remove lignin (selective delignification) whereas others degrade lignin and cellulose simultaneously	Predominantly degrade wood from deciduous trees (angiosperms), containing hardwood
Brown rot fungi	Basidiomycetes	<i>S. lacrymans</i> <i>P. betulinus</i> , <i>G. trabeum</i> , and <i>P. placenta</i>	Modify lignin by demethylation, limited aromatic hydroxylation, and ring cleavage	Preference for coniferous substrates (gymnosperms), which are softwoods
Soft rot fungi	Ascomycetes, Deuteromycetes	<i>Chaetomium</i> sp., <i>Ceratocystis</i> sp., and <i>Phialophora</i> sp.	Some lignin modification	Active generally in wet environments as well as in plant litter; attack both hardwood and softwood

^a All groups degrade cellulose and hemicellulose that serve as actual carbon and energy source.

among which 60 out of 71 (85%) occur primarily on conifers, and that they are mainly softwood degraders (Gilbertson 1980). They commonly cause decay of timber in buildings (Blanchette 2000). One of the most destructive brown rot fungi is *Serpula lacrymans*, which is well adapted to attacking timber in service and can spread rapidly on wood and traverse non-nutritional surfaces. Commonly, this type of decay has been referred to as dry rot. This term, apparently first used to describe any deterioration of dead wood or wood in service is misleading because moisture must be present for the decay to occur.

Soft rot fungi are taxonomically classified in the subdivisions, *Ascomycota* and *Deuteromycota*. They are active in environments that are too severe for white- or brown-rot fungi, generally in wet environments, but they also decompose plant litter in soils (Blanchette 1995). Soft rots are relatively unspecialized cellulolytic fungi in the genera *Chaetomium*, *Ceratocystis*, *Phialophora*, etc., that readily degrade cellulose and hemicellulose, but only modify lignin. They penetrate the secondary wall of the wood cell, forming cylindrical cavities in which the hyphae propagate. The rot is of limited extent, being closely associated with the fungal hyphae, because the cellulase enzymes do not diffuse freely through the wood.

Two distinct types of soft rot are currently recognized (Blanchette 2000). Type I is characterized by longitudinal cavities formed within the secondary wall of wood cells and Type 2 results in erosion of the entire secondary wall. The middle lamella is not degraded in contrast to cell wall erosion by white rot fungi, but may be modified in advanced stages of decay. As decay progresses, extensive carbohydrate loss occurs and lignin concentrations increase in the residual wood.

3 WHITE ROT FUNGI

White rot fungi are the only known organisms that can completely break down lignin to CO₂ and H₂O (Kirk and Farrell 1987). However, lignin cannot be degraded as a sole

source of carbon and energy by white rot fungi. Degradation of lignin enables them to gain access to cellulose and hemicellulose, which serve as their actual carbon and energy source. White rot fungi predominantly degrade wood from deciduous trees (angiosperms), containing hardwood. In a survey of 65 central European wood-decaying basidiomycetes: four were reported to only attack coniferous wood, 34 attacked angiosperms exclusively, and 27 attacked both (Rypacek 1977).

The many species that cause white rots are a heterogeneous group that may degrade greater or lesser amounts of a specific cell wall component. Some species preferentially remove lignin from wood, leaving pockets of white degraded cells that consist entirely of cellulose. This is referred to as selective delignification (Blanchette 1995; Eriksson et al. 1990). Other species degrade lignin and cellulose simultaneously which is referred to as nonselective delignification. Among the best-studied white rot fungi are *Phanerochaete chrysosporium* and *Phlebia radiata*, which degrade lignin selectively, and *Trametes versicolor* which degrades lignin nonselectively. There are fungi such as *Ganoderma applanatum* and *Heterobasidion annosum*, which are capable of both forms of degradation (Blanchette 1995; Eriksson et al. 1990). The ratio lignin–hemicellulose–cellulose decayed by a selective fungus can differ enormously and even different strains of the same species, e.g., of *P. chrysosporium* and *Ceriporiopsis subvermisporea*, may behave differently on the same kind of wood.

White rot fungi typically colonize the cell lumen and cause cell wall erosion. Degradation is usually localized to cells colonized by fungal hyphae and substantial amounts of undecayed wood remains. Progressive erosion of the cell wall occurs when components are degraded simultaneously during nonselective delignification and eroded zones coalesce as decay progresses forming large voids filled with mycelium. During selective delignification a diffuse attack of lignin occurs and white pocket or white-mottled type of rot results (Blanchette 1991; 2000).

Electron microscopy studies have revealed that lignin is degraded at some distance from the hyphae and is removed progressively from the lumen towards the middle lamella, which is also degraded (Blanchette 1984; Blanchette et al. 1987). Sheaths, often composed of β -1,3-glucans, appear to be produced during early stages of wood colonization and facilitate its degradation (Blanchette et al. 1989; Nicole et al. 1995; Ruel and Joseleau 1991). These hyphal sheaths may play an important role in transport and presentation of wood-degrading enzymes from the hyphae during the decay process, thus establishing a point of attachment to the site of degradation. The association of peroxidases to the glucan matrix is in favor of the role of the sheath as a supporting structure (Ruel and Joseleau 1991). Furthermore, the fact that the sheath was hydrolyzed during the attack demonstrated its active role both in providing the H_2O_2 necessary for peroxidase activity and in providing a mode of transport for fungal enzymes to their substrates at the surface of the wood cell wall.

3.1 Screening for Lignin Degradation by White Rot Fungi

Lignin-degrading ability is commonly evaluated by measuring $^{14}CO_2$ evolution from ^{14}C -labeled lignin preparations, such as ^{14}C -ring-labeled dehydrogenation polymerizate (DHP). The measurement of $^{14}CO_2$ evolution is the most sensitive and accurate method for testing ligninolytic activity (Eriksson et al. 1990). The evolution of $^{14}CO_2$ and the modification of DHP has been employed for determining ligninolytic activity in many white rot fungi as well as for elucidating the role of different enzymes and other constituents in lignin degradation (Boyle et al. 1992; CostaFerreira et al. 1996; Eggert et al. 1997; Hatakka 1994; Hatakka and Uusi-Rauva 1983; Hatakka et al. 1983; Hofrichter et al. 1999; Perez and Jeffries 1992; Reid and Deschamps 1991; Sethuraman et al. 1999; Silva et al. 1996; Umezawa and Higuchi 1989; Yoshida et al. 1998). Methods to study the degradation of polymeric lignin such as nuclear magnetic resonance spectroscopy have been developed (Davis et al. 1994; Gamble et al. 1994), but they are not easily amenable for detailed physiological studies with microorganisms or biochemical studies with enzymes.

A simple and reliable screening procedure that distinguishes between fungi that cause decay by selectively removing lignin and those that degrade both cellulose and lignin simultaneously has been developed involving staining of lignin with astra-blue, which stains cellulose blue only in the absence of lignin, and safranin, which stains lignin regardless of whether cellulose is present (Srebotnik and Messner 1994).

3.2 Metabolic Studies

Unfortunately, the complexity of the lignin polymer makes it difficult to study microbial ligninolysis. The lack of well-characterized model substrates that can be used to identify

ligninolytic reactions *in vivo* has been a long-standing difficulty for mechanistic studies (Crawford 1981).

Dimeric model compounds that represent the principal substructures of lignin have been used successfully to characterize the ligninolytic systems of white rot fungi. Models of this type provided some of the first evidence that *P. chrysosporium* and *T. versicolor* cleave the lignin isopropyl side chain between $C\alpha$ and $C\beta$ (Crawford 1981). Dimeric models played a large role in revealing that fungal lignin peroxidase (LIP) cleaves lignin between $C\alpha$ and $C\beta$, which represent 7% of the linkages in the lignin polymer (Gold et al. 1989; Hammel et al. 1993; Kirk and Farrell 1987). Furthermore, oxidation of a β -O-4 model compound, which represents 50–60% of the bonds within the lignin molecule demonstrated that LIP can cleave the predominance of linkages in lignin (Glenn et al. 1983; Tien and Kirk 1983a,b). The main product is the corresponding benzaldehyde. Dimeric models have also been used to detect LIP activity *in situ* in fungus-colonized wood, where extraction and conventional assay of the enzyme is technically difficult (Srebotnik et al. 1994).

Degradation of a (β -O-4)-(5-5') type trimer, arylglycerol- β -(dehydrodivanillyl alcohol) ether (I) by LIP showed that during its degradation by the enzyme, $C\alpha$ - $C\beta$ cleavage, β -O-4 bond cleavage, and β -etherified aromatic ring (B-ring) opening products were formed (Umezawa and Higuchi 1989; Yokota et al. 1991). The results showed that the B-ring of substrate (I), which must be sterically hindered more than those of arylglycerol- β -guaiacyl and arylglycerol- β -(2,6-dimethoxyphenyl) ethers was oxidized by LIP (Umezawa and Higuchi 1989; Yokota et al. 1991).

Dimeric lignin model compounds have the disadvantage of low molecular weight. Unlike lignin, they can be taken up and metabolized intracellularly by microorganisms, which can make it difficult to determine whether the degradation products observed really reflect ligninolytic activity (Crawford 1981). Ideally, lignin model compounds should be macromolecular like lignin, but to facilitate product analysis, they should have simpler structures than those of the natural polymer. For this reason dimeric lignin compounds attached to a polymer backbone such as polystyrene and polyethylene glycol have been developed (Kawai et al. 1995).

4 LIGNINOLYTIC SYSTEM OF WHITE ROT FUNGI

In order to degrade lignin, the white rot fungi have developed an unspecific ligninolytic system consisting of peroxidases and laccases (phenol oxidases; LAC), which degrade lignin in an oxidative process (Hatakka 1994). The peroxidases are heme-containing enzymes and require the presence of hydrogen peroxide (H_2O_2) to oxidize lignin and lignin-related compounds. Three types of peroxidases have been discovered in white rot fungi: LIP, manganese peroxidase (MnP), and more recently versatile peroxidase (VP)

Table 2 Enzymes and their roles in lignin degradation by white rot fungi

Enzyme	Cofactors or mediators	Role in lignin degradation	References
LIP	H ₂ O ₂ , 3,4-dimethoxybenzyl alcohol (veratryl alcohol), 3,4-dimethoxycinnamic acid, 1,2-dimethoxybenzene, and 2-chloro-1,4-dimethoxybenzene	Direct and mediated oxidation of phenolic and nonphenolic lignin structures, respectively. This results in cleavage of C α -C β , β -O-4 and aryl-C α bonds, aromatic ring opening, hydroxylation, and demethoxylation	For review see Tuor et al. (1995)
MnP	H ₂ O ₂ , Mn ²⁺ , organic acids as chelators, and unsaturated fatty acids	Mn ²⁺ oxidized to Mn ³⁺ ; chelated Mn ³⁺ oxidizes phenolic lignin structures and subsequently, alkyl-phenyl cleavage, C α -C β cleavage, or benzylic carbinol oxidation may result; nonphenolic moieties may be co-oxidized when MnP peroxidizes unsaturated fatty acids	For review see Tuor et al. (1995)
VP	H ₂ O ₂ , veratryl alcohol, Mn ²⁺ , organic acids as chelators, and unsaturated fatty acids	Still unknown	Camarero et al. (1999), Mester and Field (1998), and Ruiz-Duenas et al. (1999, 2001)
LAC	O ₂ , 3-hydroxyanthranillic acid, and hydroxybenzotriazole	Phenolic lignin structures are oxidized to phenoxy radicals and subsequently, alkyl-phenyl cleavage or C α -C β cleavage may result. Dimethoxylation of several lignin model compounds has been witnessed; nonphenolic lignin structures may be oxidized only in the presence of mediators	For review see Tuor et al. (1995)
CDH	Use electron acceptors including quinones, phenoxy radicals, Fe ³⁺ , and Cu ²⁺ to generate lactones from various substrates	Reduces aromatic radicals preventing repolymerization, demethoxylation, or hydroxylation of nonphenolic lignin, and reduction of precipitated MnO ₂	For review see Henriksson et al. (2000)
GLOX	Glyoxal and methyl glyoxal	Glyoxal oxidized to glyoxylic acid and concomitant production of H ₂ O ₂	Zhao and Janse (1996)
AO	Aromatic alcohols (anisyl and veratryl alcohol)	Aromatic alcohols oxidized to aldehydes with concomitant production of H ₂ O ₂	Zhao and Janse (1996)
Other H ₂ O ₂ generating enzymes	Many organic compounds	O ₂ reduced to H ₂ O ₂	Urzua et al. (1998) and Zhao and Janse (1996)

Table 3 Selective white rot fungi and their reported ligninolytic enzymes

Microorganism	Enzymes				References
	LIP	MnP	Lac	VP	
<i>P. brevispora</i>	✓	✓	✓		Arora and Gill, (2001), Perez and Jeffries (1990), and Ruttimann et al. (1992)
<i>P. radiata</i>	✓	✓	✓		Niku-Paavola et al. (1988) and Vares et al. (1995)
<i>P. tremellosa</i>	✓	✓	✓		Bonnarme and Jeffries (1990) and Hatakka et al. (1992, 1993)
<i>I. lacteus</i>	✓	✓	✓		Novotny et al. (2000) and Rothschild et al. (2002)
<i>B. adusta</i>	✓	✓	✓	✓	Heinfling et al. (1998a,b), Kimura et al. (1991), and Nakamura et al. (1999)
<i>Bjerkandera</i> sp. strain BOS55	✓	✓	✓	✓	Mester and Field (1998), Mester et al. (1995), and ten Have et al. (1998a)
<i>T. versicolor</i>	✓	✓	✓		Dodson et al. (1987), Fahraeus and Reinhammar (1967), and Johansson and Nyman (1987)
<i>P. chrysosporium</i>	✓	✓			Couto et al. (1999), Glenn et al. (1983), Leisola et al. 1987, and Tien and Kirk (1983b)
<i>P. pini</i>	✓	✓			Bonnarme and Jeffries (1990)
<i>T. pruinosum</i>	✓	✓			Waldner et al. (1988)
<i>P. ochraceofulva</i>	✓		✓		Hatakka (1994) and Vares et al. (1993)
<i>J. separabilima</i>	✓		✓		Vares et al. (1992)
<i>P. ostreatus</i>		✓	✓	✓	Cohen et al. (2001), Sannia et al. (1986), and Waldner et al. (1988)
<i>P. eryngii</i>		✓	✓	✓	Camarero et al. (1999), Heinfling et al. (1998b), Munoz et al. (1997), and Ruiz-Duenas et al. (1999)
<i>P. sajor-caju</i>		✓	✓		Bourbonnais and Paice (1989), Buswell et al. (1996), and Fukuzumi 1987
<i>D. squalens</i>		✓	✓		Perie and Gold (1991) and Perie et al. (1998)
<i>L. edodes</i>		✓	✓		Forrester et al. (1990) and Leatham and Stahmann (1984)
<i>G. lucidum</i>		✓	✓		D'Souza et al. (1999)
<i>P. tigrinus</i>		✓	✓		Golovleva et al. (1993) and Leontievsky et al. (1994)
<i>R. lignosus</i>		✓	✓		Galliano et al. (1991)

(Camarero et al. 1999; Mester and Field 1998; Ruiz-Duenas et al. 1999; 2001). Laccases are multicopper phenol oxidases, which oxidize phenols and aromatic amines. Rather than H_2O_2 , these enzymes utilize dioxygen (O_2) as an oxidant, reducing it by four electrons to water (Call and Mucke 1997). The enzymes involved in lignin degradation along with the roles they play are given in Table 2.

White rot fungi have been classified according to the ligninolytic enzymes they express. Whereas Hatakka suggested that they can be classified into three categories (Hatakka 1994), Tuor et al. classified them into five categories as follows (Tuor et al. 1995): (a) White rot fungi expressing LIP, MnP, and LAC, (b) white rot fungi simultaneously producing MnP and LAC, but not LIP, (c) white rot fungi producing LIP and either MnP or LAC, (d) white rot fungi reported to produce LIP without MnP or LAC, (e) a group which is incompletely characterized and in which neither of the oxidative enzymes have been identified.

However, the continual discovery of enzymes in different white rot fungi species means that classifications of this type are subject to ongoing changes. In particular, the recent discovery of the novel VP in *Pleurotus eryngii* and

Bjerkandera adusta (Camarero et al. 1999; Mester and Field 1998; Ruiz-Duenas et al. 1999; 2001), warrants re-evaluation of the classification scheme. Selective white rot fungi and the ligninolytic enzymes that they produce are given in Table 3.

4.1 Lignin Peroxidase

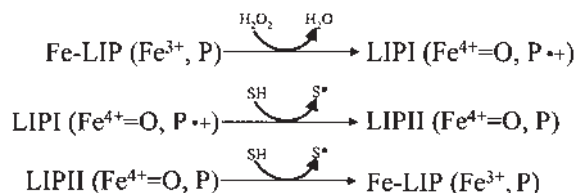
4.1.1 Properties

Lignin peroxidase is secreted as a series of glycosylated isoenzymes with pI's ranging from 3.2 to 4.0 and molecular masses ranging from 38 to 43 kDa, with each isoenzyme containing 1 mol heme per mole of protein (Farrell et al. 1989; Gold and Alic 1993; Leisola et al. 1987). It possesses a higher redox potential and a lower pH optimum than that of any other isolated peroxidase or oxidase (Call and Mucke 1997; Hammel et al. 1986; Kersten et al. 1990). Like other peroxidases, LIP is capable of oxidizing most phenolic compounds through the generation of phenoxy radicals. However, due to its exceptionally high redox potential and low pH optimum, it is able to oxidize nonphenolic aromatic

substrates, typically not oxidized by other peroxidases including the nonphenolic phenylpropanoid units of lignin (Hammel et al. 1986; Hatakka 1994; ten Have et al. 1998b; Kersten et al. 1990). Stable cation centered radicals formed during the oxidation of nonphenolic aromatic nuclei may serve as redox mediators for LIP-catalyzed oxidations, effectively extending the substrate range. Reactions catalyzed by LIP include benzyl alcohol oxidations, side-chain cleavages, ring-opening reactions, dimethoxylations, and oxidative dechlorinations. The ability of LIP to attack such a variety of linkages suggests that it plays a key role in lignin degradation.

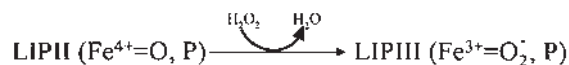
4.1.2 Catalytic Cycle

The catalytic cycle of LIP is similar to that of other peroxidases (Renganathan and Gold 1986; Tien et al. 1986).



Reaction of native ferric enzyme [Fe-LIP; Fe³⁺, P (porphyrin)] with H₂O₂ yields LIP-compound I (LIPI) a complex of high valent oxo-iron and porphyrin cation radical (Fe⁴⁺=O, P^{•+}). One-electron-oxidation of a reducing substrate (SH) by LIPI yields a radical cation (S[•]) and the one-electron-oxidized enzyme intermediate, LIP-compound II (LIPII; Fe⁴⁺=O, P). A single one-electron oxidation of a second substrate molecule returns the enzyme to Fe-LIP completing the catalytic cycle.

However, in the absence of suitable reducing substrate or, at high H₂O₂ concentrations, LIPII is further oxidized by H₂O₂ to LIP-compound III (LIPIII; Fe³⁺=O₂⁻, P), a species with limited catalytic activity.

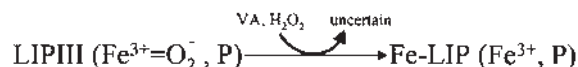


Lignin peroxidase is unique from other peroxidases in that it exhibits an unusually high reactivity between LIPII and H₂O₂ (Cai and Tien 1989; 1992; Wariishi and Gold 1990; Wariishi et al. 1990). Since, LIPIII is inactivated rapidly in the presence of excess H₂O₂, if it is not rapidly reverted to the native state, the enzyme has a "suicidal" tendency.

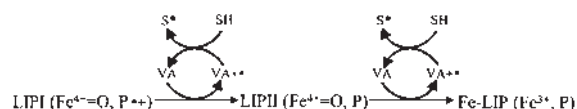
4.1.3 Role of Veratryl Alcohol During LIP-catalyzed Oxidation

The LIPIII has been shown to readily return to the native ferric state in the presence of H₂O₂ and veratryl alcohol (3,4-dimethoxybenzyl alcohol; VA) (Barr and Aust 1994; Cai and Tien 1989; 1992; Wariishi and Gold 1990). Ligninolytic cultures of *P. chrysosporium* normally produce VA, one of

the physiological roles of which is believed to protect LIP from H₂O₂-dependent inactivation by reverting LIPIII to the native state. This is of particular significance, since during oxidation of certain chemicals such as phenols, LIPIII has been shown to accumulate, indicating that they are either poor substrates for LIPII or they lack the ability to revert LIPIII to the native state (Chung and Aust 1995; Harvey and Palmer 1990). Thus, oxidation of such chemicals is inefficient at high H₂O₂ concentrations.



Veratryl alcohol has also been shown to act as a charge-transfer mediator in LIP catalyzed reactions (Goodwin et al. 1995; Harvey et al. 1986). During the catalytic cycle of LIP, VA is oxidized to VA cation radical (VA^{•+}), which in the presence of suitable reducing substrate is reduced back to VA and ready for another LIP catalyzed charge-transfer reaction. The roles played by VA in the catalytic cycle of LIP are highlighted.



Low molecular weight redox mediators such as VA are believed to be essential for degradation of lignin, since theoretical and electron microscopic studies have demonstrated that enzymes as large as peroxidases and LAC cannot have direct contact with lignin, since they appear to be too large for the penetration of the cell wall or the middle lamella (Call and Mucke 1997). Pine decayed by wood rotting fungi was infiltrated with a concentrated culture filtrate of the white rot fungus *P. chrysosporium* and labeled for LIP by postembedding immunoelectron microscopy. This method demonstrated that enzymatic attack on pine by LIP and presumably also other enzymes of the same size is restricted to the surface of the wood cell wall (Srebotnik and Messner 1988).

Measurements of the stability of VA^{•+} have led to the suggestion that it would be unable to act as a diffusable oxidant without some form of stabilization (Candeias and Harvey 1995; Khindaria et al. 1996). It was suggested that a LIP-VA^{•+} complex acts as the redox partner, with the cation radical being stabilized by a protein microenvironment of acidic character (Khindaria et al. 1996).

Nonphenolic aromatic compounds other than VA, such as 3,4-dimethoxycinnamic acid, 1,2-dimethoxybenzene, and 2-chloro-1,4-dimethoxybenzene have also been shown to be capable of mediating oxidation (Teunissen and Field 1998; Ward et al. 2002). The mediation phenomenon appears to be driven by the difference in the oxidation potential (OP) and site-binding affinity of the mediators (possessing higher OP values and higher affinity) and the target substrates (possessing lower OP values and lower affinity) (Ward et al. 2002).

4.2 Manganese Peroxidase

4.2.1 Properties

Manganese peroxidase exists as a series of glycosylated isozymes with pI s ranging from 4.2 to 4.9 and molecular masses ranging from 45 to 47 kDa. Similar to LIP, each isozyme contains 1 mol of iron per mole of protein (Leisola et al. 1987; Paszczynski et al. 1986). To date, five isozymes have been detected in *P. chrysosporium* MP-1 (Kirk and Cullen 1998).

4.2.2 Catalytic Cycle

Manganese peroxidase, like LIP has the same catalytic cycle as other peroxidases, involving a two-electron oxidation of the heme by H_2O_2 , followed by two subsequent one-electron reductions to the native ferric enzyme. The primary reducing substrate in the MnP catalytic cycle is Mn^{2+} , which efficiently reduces compound I and compound II, generating Mn^{3+} , which then serves to oxidize phenols to phenoxy radicals (Hatakka 1994).

Whereas it has been shown that cation radicals of aromatic substrates such as that of VA maintain the active form of LIP by oxidatively converting compound III to the native enzyme and preventing H_2O_2 -dependent inactivation (Barr and Aust 1994), Mn^{3+} has similarly been shown to convert MnP compound III to native enzyme (Timofeevski et al. 1998). Additionally, Mn^{2+} also reactivated compound III and although this reaction was slower, it could prevent compound III accumulation when excess Mn^{2+} was present.

4.2.3 Role of MnP in Lignin Degradation

In many fungi, MnP is thought to play a crucial role in the primary attack on lignin, because it generates Mn^{3+} , a strong diffusible oxidant able to penetrate the small "molecular pores" between cellulose microfibrils, which precludes the action of LIP because of steric hindrances (Flournoy et al. 1993). Organic acids, such as oxalate (Kuan and Tien 1993), and fumarate and malate (Hofrichter et al. 1999), which are also produced by white rot fungi, chelate Mn^{3+} . These stable complexes then deliver the oxidizing power. Although MnP does not oxidize nonphenolic lignin structures during normal turnover, these structures have been shown to be slowly co-oxidized when MnP peroxidizes unsaturated fatty acids (Jensen et al. 1996; Kapich et al. 1999a). Bao et al. described the oxidation of a nonphenolic lignin model by a lipid peroxidation system that consisted of *P. chrysosporium* MnP, Mn^{2+} , and unsaturated fatty acid esters (Bao et al. 1994). Substrate oxidation occurred via benzylic hydrogen abstraction and it was suggested that this process might enable the white rot fungi to accomplish the initial delignification of wood. The importance of Mn^{2+} and the involvement of lipid peroxidation in depolymerization and mineralization of ^{14}C -labeled, polyethylene glycol linked, β -O-4 lignin model compound by *C. subvermispora* in wood block cultures and

defined medium has been demonstrated (Jensen et al. 1996). Lipid peroxidation has been suggested as the mechanism involved in the oxidation of the nonphenolic lignin structures by white rot fungi that do not produce LIP.

4.3 Versatile Peroxidase

A heme peroxidase different from other microbial, plant, and animal peroxidases, termed VP has recently been discovered in *Pleurotus* and *Bjerkandera* species (Camarero et al. 1999; Mester and Field 1998; Ruiz-Duenas et al. 1999; 2001). The VP is characterized by catalytic properties of MnP and LIP. The enzyme exhibits high affinity for Mn^{2+} , hydroquinones, and dyes, and also oxidizes VA, dimethoxybenzene, and lignin dimers (Ruiz-Duenas et al. 2001). Molecular models show a Mn^{2+} -binding site formed by three acidic residues near the heme internal propionate accounting for the ability of VP to oxidize Mn^{2+} (Ruiz-Duenas et al. 1999). Concerning aromatic substrate oxidation, VP shows a putative long-range electron transfer pathway from an exposed tryptophan to heme, similar to that postulated in LIP (Ruiz-Duenas et al. 2001). Mutagenesis and chemical modification of this tryptophan and the acidic residues forming the Mn^{2+} -binding site confirmed their role in catalysis.

4.4 Laccase

4.4.1 Properties

Laccases are multicopper phenol oxidases that oxidize phenols and aromatic amines. Rather than H_2O_2 , these enzymes utilize O_2 as an oxidant, reducing it by four electrons to H_2O (Call and Mucke 1997). LACs are generally larger than peroxidases, having molecular weights of approximately 60 kDa and above (Call and Mucke 1997). As with other extracellular enzymes, LACs are glycosylated.

4.4.2 Role of LAC in Lignin Degradation

Laccase oxidizes phenolic lignin model compounds directly (Kawai et al. 1988). Although it is a phenoloxidase and its redox potential is too low to directly oxidize the nonphenolic components of lignin, it has been shown to degrade lignin efficiently in the white rot *Pycnoporus cinnabarinus*, which does not produce MnP or LIP (Eggert et al. 1997). To overcome the redox potential barrier, *P. cinnabarinus* produces a metabolite, 3-hydroxyanthranilate that can mediate the oxidation of nonphenolic substrates by LAC (Eggert et al. 1996). It is believed that natural mediators, such as 3-hydroxyanthranilic acid in the white rot *P. cinnabarinus* act as diffusible lignin-oxidizing agents. In the presence of the mediators 3-hydroxyanthranilic acid, hydroxybenzotriazole, or 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), LAC has been shown to oxidize nonphenolic lignin, VA, and PAH (Bourbonnais et al. 1995; Collins et al. 1996; Eggert et al. 1996; Majcherczyk et al. 1998; 1999).

4.5 H₂O₂ Generating Enzymes

In addition to the enzymes mentioned earlier, the ligninolytic system of white rot fungi includes extracellular H₂O₂ generating enzymes, essential for peroxidase activity. To date, a number of oxidase enzymes have been shown to lead to the production of H₂O₂, including glyoxal oxidase (GLOX), glucose 1-oxidase, methanol oxidase, and aryl-alcohol oxidase (AO) (Zhao and Janse 1996). White rot fungi that lack H₂O₂ generating oxidases may rely on the oxidation of physiological organic acids such as oxalate and glyoxalate, which indirectly results in H₂O₂ production (Urzua et al. 1995).

4.6 Cellobiose Dehydrogenase

Cellobiose dehydrogenase (CDH) is an extracellular enzyme produced by many white rot fungi (Henriksson et al. 2000). It oxidizes soluble cellodextrins, mannodextrins, and lactose efficiently to their corresponding lactones by a ping-pong mechanism using a wide spectrum of electron acceptors including quinones, phenoxy radicals, Fe³⁺, Cu²⁺, and triiodide ion. The function of CDH is not obvious, but *P. chrysosporium* produces relatively high levels of the enzyme, approximately 0.5% of the secreted protein on a mass basis, suggesting it plays an important role. Many functions have been suggested for CDH, some of which are related to cellulose degradation, but in the current context only those that are relevant to lignin biodegradation will be discussed. It has been suggested that CDH reduces aromatic radicals formed by ligninolytic enzymes, thereby preventing their repolymerization and supporting lignin degradation (Ander et al. 1990; Temp and Eggert 1999). In addition, it has been shown that CDH can generate hydroxyl radicals (OH•) in a fenton type reaction, which can modify cellulose, hemicellulose, and lignin (Henriksson et al. 1995; Wood

1994). The modification of lignin by OH• radicals produced by CDH may result in hydroxylation of nonphenolic structures to phenolic ones, facilitating MnP and LAC action and suggesting that the enzymes may form a pathway in lignin biodegradation (Hilden et al. 2000). It has also been suggested that CDH may support MnP by reducing precipitated MnO₂, which is common in rotten wood to Mn²⁺ or Mn³⁺. It might also aid MnP by producing cellobionic acid, which should complex Mn³⁺ (Roy et al. 1994).

4.7 Reactive Oxygen Species

Reactive oxygen species and the suggested roles that they play in lignin degradation are given in Table 4.

The production of OH• radicals by white rot fungi is well documented (Barr et al. 1992; Kutsuki and Gold 1982; Tanaka et al. 1999a,b). OH• radicals are very reactive and can attack the subunits of lignin by both abstracting aliphatic Cα-hydrogens and by adding to aromatic rings (Hammel et al. 2002). Typical reactions of OH• radical with the major arylglycerol-β-aryl ether structure of lignin can result in demethoxylation, β-O-4 cleavage, hydroxylation, or Cα-oxidation (Hammel et al. 2002). The oxidation of lignin by OH• radicals, therefore, results in diverse reactions, some of which are expected to degrade the polymer. However, it remains unclear whether any wood decay fungus uses OH• radical to oxidize lignin. Hydroxylation of both phenolic and nonphenolic lignin resulting in new phenolic substructures on the lignin polymer may make it susceptible to attack by LAC or MnP (Hilden et al. 2000; Tanaka et al. 1999a).

If white rot fungi produce OH• radicals, then it is also necessary to consider the effects that peroxy (ROO•) and hydroperoxy (HOO•) radicals have on lignin, since both of these ROS are expected as secondary radicals when OH• radicals oxidize wood polymers (Hammel et al. 2002). Manganese peroxidase of white rot fungi peroxidizes

Table 4 Reactive oxygen species and their role in lignin degradation by white rot fungi

Reactive oxygen species	Role in lignin degradation	References
Hydroxyl radicals (OH•)	Demethoxylation, β-O-4 cleavage, hydroxylation, or Cα-oxidation of nonphenolic structures; hydroxylation of nonphenolic lignin results in the formation of phenolic structures, making it susceptible to attack by MnP and LAC	Hammel et al. (2002), Hilden et al. (2000), and Tanaka et al. (1999a)
Peroxy radicals (ROO•)	Oxidation of nonphenolic lignin. Cα–Cβ and β-O-4 cleavage	Hammel et al. (2002)
Superoxide radicals (O ₂ ^{•−})	Production of H ₂ O ₂ by dismutation; Mn ²⁺ oxidation to Mn ³⁺ ; production of OH• radicals through iron-catalyzed Haber–Weiss reaction; by reacting with phenoxyl radicals produced from lignin model compounds, it can result in oxidative degradation being favored over coupling reactions	Archibald and Fridovich (1982), Barr et al. (1992), and Gierer et al. (1994)

unsaturated fatty acids, which results in the formation of ROS that include ROO• (Moen and Hammel 1994). ROO• radical-generating systems including MnP in the presence of unsaturated fatty acids were shown to oxidize a nonphenolic β -O-4-linked lignin model dimer to products indicative of hydrogen abstraction (Kapich et al. 1999b). Since, white rot fungi do produce extracellular lipids (Enoki et al. 1999), the formation of ROO• radicals by an MnP dependent mechanism and their involvement in lignin degradation does seem reasonable. The involvement of ROO• radicals in the chemical consumption of 1-(3',4'-dimethoxyphenyl) propene (DMPP) by LIP was confirmed by using the well-known ROO• radical reductant Mn^{2+} (ten Have et al. 2000). This metal ion severely inhibited the DMPP consumption rate under air, but did not affect the lower enzymatic DMPP consumption rate under N_2 . In the absence of O_2 , the $C\alpha-C\beta$ cleavage of DMPA to veratryl aldehyde was strongly inhibited and side-chain coupling products (dimers) were formed instead. As a whole, these results suggest that during LIP-catalyzed oxidation of aromatic substrates, O_2 is responsible for the formation of reactive ROO• intermediates, which can directly react with other substrate molecules and thereby accelerate consumption rates and also prevents coupling reactions by lowering the pool of carbon-centered radicals accumulating during LIP catalysis.

In contrast to OH• and ROO•, superoxide anion radicals ($O_2^{\bullet-}$) are unable to oxidize lignin units. However, $O_2^{\bullet-}$ produced by white rot fungi can participate in the production of H_2O_2 via both dismutation ($2O_2^{\bullet-} + 2H^+ = H_2O_2 + O_2$) and Mn^{2+} oxidation with concomitant production of Mn^{3+} ($O_2^{\bullet-} + Mn^{2+} + 2H^+ = H_2O_2 + Mn^{3+}$) (Archibald and Fridovich 1982). They can also be involved in HO• production through the iron-catalyzed Haber–Weiss reaction ($O_2^{\bullet-} + H_2O_2 = HO\bullet + HO^- + O_2$) (Barr et al. 1992). Furthermore, by reacting with phenoxyl radicals produced from lignin model compounds, they can result in oxidative degradation being favored over coupling reactions (Gierer et al. 1994).

One potential source of $O_2^{\bullet-}$ evolves from the cleavage of oxalate via oxalate decarboxylase (ten Have and Teunissen 2001). Oxalate is produced as a major aliphatic acid by white rot fungi (Makela et al. 2002). Its decomposition results in the formation of CO_2 and the formate anion radical ($CO_2^{\bullet-}$), which is further oxidized by O_2 to give CO_2 and $O_2^{\bullet-}$ or HOO•. Subsequent dismutation of $O_2^{\bullet-}$ resulting in the formation of H_2O_2 indicates that oxalate may serve as a passive sink for production of the latter. Both LIP and MnP are also capable of decomposing oxalate in the presence of VA and Mn^{2+} , respectively (Akamatsu et al. 1990; Shimada et al. 1994) and indeed other organic acids (Hofrichter et al. 1998; Urzua et al. 1998). These reactions account for the observed oxidation of phenol red and kojic acid by MnP in the presence of Mn^{2+} , without exogenous addition of H_2O_2 (Kuan and Tien 1993; Urzua et al. 1995). The reduction of $VA^{+\bullet} +$ or Mn^{3+} by oxalate suggests that as long as oxalate coexists with LIP and MnP, it would inhibit lignin degradation. Indeed, oxalate has been shown to strongly

reduce the rate of lignin mineralization in ligninolytic cultures of white rot fungi (Akamatsu et al. 1990; Ma et al. 1992; Shimada et al. 1994).

5 PHYSIOLOGICAL REQUIREMENTS FOR LIGNIN DEGRADATION BY WHITE ROT FUNGI

Lignin is unable to serve as the sole carbon and energy source for white rot fungi. In order for lignin degradation to proceed, white rot fungi require an additional more readily utilizable source of carbon (Ander and Eriksson 1975; Kirk et al. 1976). It has been hypothesized that the normal mode for white rot fungi to degrade wood is by simultaneous attack of polysaccharides and lignin (Boominathan and Reddy 1992). Degradation of cellulose and hemicellulose provides glucose for the fungus and when the flow of sugar ceases, the fungus starves and thereby goes from primary to secondary metabolism.

White rot fungi degrade lignin at the onset of the secondary growth phase, when utilizable nutrients are depleted and primary fungal growth ceases (Bonnarme et al. 1991). However, it should be considered that the concepts of primary and secondary metabolism might be an oversimplification regarding lignin degradation during natural conditions where fungal growth and lignin degradation can occur at the same time. Carbon, nitrogen, and manganese are all critical nutritional variables in triggering secondary metabolism and the production of ligninolytic enzymes including LIP and MnP by *P. chrysosporium* and other white rot fungi (Bonnarme et al. 1991). Considering that the nitrogen content of wood is very low (C/N 350-500/1) (Cowling and Merrill 1966), it is no surprise that nitrogen plays an important role in growth and metabolism (Buswell and Odier 1987; Kirk and Farrell 1987). In *P. chrysosporium*, limitation of nitrogen, carbon, or sulfur (Jeffries et al. 1981) can trigger secondary metabolism and lignin degradation. Fungal degradation of lignin in wheat straw was affected by the amount of nitrogen (NH_4NO_3), which appears to repress lignin degradation, by most fungi on this substrate (Zadrazil and Brunnert 1980). However, nutrient nitrogen only had a moderate influence on lignin mineralization by *T. versicolor* and almost no influence on *P. ostreatus* and *Lentinus edodes* (Leatham and Kirk 1983). In general, nitrogen repression of lignin degradation in white rot fungi is common but it is not always a rule.

The ligninolytic activity in *P. chrysosporium* is also triggered in cultures where carbon becomes limiting. The activity appears when the carbon source is depleted, and this activity is associated with a decrease of mycelial dry weight. The amount of lignin degraded depends on the amount of carbohydrate provided, which in turn determines the amount of mycelium produced during primary growth (Jeffries et al. 1981).

6 POTENTIAL APPLICATIONS OF WHITE ROT FUNGI

6.1 Upgrading Agricultural Wastes for Animal Feed

The direct use of lignocellulosic residues as ruminant animal feed, or as a component of such feeds, represents one of its oldest and most widespread applications and, as such, it plays an important role in the ruminant diet. The lignocellulose complex in straw and other plant residues is degraded very slowly by ruminants, because of the physical and chemical barrier imposed by lignin polymers, preventing free access of hydrolytic enzymes, such as cellulases and hemicellulases to their substrates. Normally, the rate of decay of plant debris is proportional to its lignin content. Delignification of straw by white rot fungi seems to be the most promising way of improving its digestibility (Kamra and Zadrazil 1986; Streeter et al. 1982; Zadrazil and Reinger 1988). The role of fungi in agricultural waste conversion by different fungi species has been recently reviewed (Cohen and Hadar 2001).

6.2 Biopulping

The objective of pulping is to extract cellulose fibers from plant material, generally hard or soft wood trees. Mechanical and chemical pulping are usually employed for this process. However, a biological approach involving white rot fungi could replace environmentally unfriendly chemicals (e.g., chlorine) save on mechanical pulping energy costs and improve the quality of pulp and the properties of paper (Breen and Singleton 1999). The ligninolytic enzymes of white rot fungi selectively remove or alter lignin and allow cellulose fibers to be obtained. Recent data suggest that biopulping has the potential to be an environmentally and economically feasible alternative to current pulping methods (Akhtar et al. 2000; Scott et al. 1998).

6.3 Mushroom Production

Several of the white rot fungi that can utilize lignocellulose are edible mushrooms. They have been successfully cultivated at a commercial level worldwide using lignocellulosic wastes as the main substrate (Wood and Smith 1987). *Agaricus bisporus*, known as the "button mushroom," *L. edodes* known as "Shiitake," and *P. ostreatus*, known as the "oyster mushroom" are just three examples of this agricultural-biotechnological crop. Bioconversion of lignocellulosic residues through mushroom cultivation also offers the potential for converting these residues into protein-rich palatable food, reducing the environmental impact of the wastes.

7 CONCLUSIONS

Lignin degradation plays a central role in the earth's carbon cycle, since it hinders decomposition of the most renewable carbon sources, cellulose and hemicellulose. White rot fungi able to cause complete wood decay are the most efficient lignin degraders known and they are perhaps nature's major agents for recycling the carbon of lignified tissues. Considerable research has been devoted to understanding the chemistry, biochemistry, and genetics underlying the degradation of lignin by these fungi. Many enzymes and several small molecules including reactive oxygen species and organic acids have been described as playing a role in lignin degradation by white rot fungi in nature. However, a major breakthrough is still required to understand the relative contribution of the individual components of the ligninolytic system and the synergism that exists between them. Overcoming the difficulties associated with studying the degradation of such a complex polymer could enable this. Obviously, progress in these directions would have far reaching implications and could be of considerable aid in developing the many potential biotechnological applications that have been proposed for the ligninolytic system of white rot fungi.

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Biom mineralization of Heavy Metals

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1 INTRODUCTION

For well over a century, metal-contaminated industrial wastewaters have been released into the environment by industry, agriculture, sewage treatment, and mining operations worldwide. Since the WWII era, the nuclear fuel cycle has contributed an additional and unique waste burden of uranium and other radioactive metals. Metal ions, unlike most organic chemicals, can persist in the environment indefinitely, posing threats to organisms which are exposed to them (Volesky and Holan 1995). Governmental control of such discharges has only been energetically regulated in the past two or three decades. Many toxic inorganic chemicals have, over time, accumulated in soils, sediments, and impoundments throughout the world. Metal-bearing liquid wastes may be of known and predictable composition if generated by a single industry, e.g., electroplating wastewaters, or in other cases may be a heterogeneous mix of many dissolved metal ions and organic compounds at various pH values and ionic strengths, with colloidal and particulate matter present as well. Governments are now regulating this problem by mandating preventative actions and forcing industries and laboratories and other waste generators to intercept toxic metals before they are discharged. Most heavy metal containing wastewaters are treated using remediation technologies that have been borrowed primarily from the unit operations of the chemical industry which rely on a mixture of physical and chemical processes (Table 1) to render the metal ion contaminants less toxic or more easily handled. Unfortunately the chemical form of the converted metal (e.g., a gelatinous precipitate) is itself often in need of careful and expensive disposal, and conventional treatment becomes less efficient and more expensive when metal ion

concentrations fall into the 1–10 mg/l range. Table 2 provides a listing of discharge limits of metal finishing wastewaters in the United States, and as such represents goals that must be met by any new technology or existing technologies. Discharge limits for municipal wastewater treatment plants in the United States are much stricter than those listed in Table 2. Influent levels of Cu^{2+} in wastewaters arriving at municipal sewage treatment plants range from 100 to 250 $\mu\text{g/l}$, but effluent levels of 6–25 $\mu\text{g/l}$ are expected to be attained under new U.S. Environmental Protection Agency guidelines (Amer 1998). Regulations governing aqueous metal discharges in the United Kingdom have been reviewed by Forster and Wase (1997) who also discussed the toxic biological effects of several of the important heavy metals. A genuine need now exists for new and certainly more cost-effective technologies to replace or supplement the physico-chemical approaches currently in use for removing metal contamination at existing sites and for preventing future contamination of natural waters by heavy metals. There is hope that biotechnology may provide new insights to solving these problems (Crusberg et al. 1994; 1996; Gretsky 1994).

Biotechnology has been successfully exploited as a remedy for many types of discharges of organic wastes and for in situ bioremediation of sediments at contaminated sites and in fact is the preferred remedy for many instances (Alexander 1999). There is hope that some of the physiological processes and genetic adaptations that protect organisms against toxic metals and other inorganic contaminants can be identified and exploited for the removal and recovery of those metals from aqueous waste streams (Crusberg et al. 1991; 1996; Hartley et al. 1997; Nies 1999; Ow 1996; 1997). Systems which use renewable biomass to extract metal ions from solutions may be an environmentally friendly alternative to physico-chemical

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Table 1 Physicochemical processes for heavy metal wastewater treatment

Process	Detrimental factors
Membrane separation	Expensive, durability of membranes
Liquid–liquid extraction	Limited applications
Carbon adsorption	Expensive adsorbent, requires regeneration
Ion exchange	Expensive adsorbent, requires regeneration
Electrolytic treatment	Limited applications
Precipitation	Forms sludges, often very gelatinous
Coagulation/flocculation	Forms hydrated sludges
Chemical reduction	Limited applications
Flotation	Forms hydrated materials
Vitrification	Very expensive, landfill required
Evaporation	Energy intensive
Crystallization	Time dependent, landfill required

(From U.S.E.P.A., 1991; Smith et al. 1995.)

processes and will be considered for their ability to serve as biotrap for metals (chiefly as ions). This discussion will center on heterotrophic microorganisms, primarily yeast and fungi as possible candidates for development as heavy metal biosorbents or “biotraps.” Biotraps in this review are defined as any organism (living or nonliving) or component of an organism, which can alter the form of, or bind with a toxic metal or metal ion allowing its removal and recovery from a waste stream, or rendering it harmless.

2 POTENTIAL OF FUNGAL BIOTECHNOLOGY

It has been recognized for decades that many microorganisms have a physiology, which allows them to act on

Table 2 Pretreatment standards based upon Best Practical Control Technology (BPT) for existing sources of metal finishing wastewaters. Discharges for releases of 10,000 or more ga (38,000 l) a day in the United States. (40 CFR (Code of Federal Regulations of the U.S.) 413)

Metal ion	Maximum for any 1 day	Average daily value for 30 consecutive days
Cu	3.38 mg/l	2.07 mg/l
Ni	3.98	2.38
Cr	2.77	1.71
Zn	2.61	1.48
Pb	0.69	0.43
Cd	0.69	0.26
Ag	0.43	0.24

toxic metal ions and to alter them in such a way that renders those ions less harmful. In fact, genetic adaptation to survival of microorganisms in heavy metal-rich environments is not at all unusual in nature (Nies 1999; Ow 1996).

One aim of biotechnology is to put that special physiology to use and produce a commercial product (cell or cell component) for remediating heavy metal contamination. Biological materials that can accomplish this mission may be therefore termed biotraps and can be grouped into three major categories based on function:

- a. Biosorption or the process by which individual cells or cell components bind or take up metals
 - a. Adsorption of ions onto a surface, including ion exchange and complexing with ligands, and
 - b. Uptake into the cell and sequestration by cytoplasmic components,
- b. Transformation by oxidation/reduction into a less toxic or volatile form, and
- c. Precipitation by forming insoluble salts, or by a combination of these processes.

This review discusses general heavy metal bioremediation processes rather than have to deal with each group of organisms individually.

3 FUNGAL-MEDIATED HEAVY METAL REMOVAL FROM AQUEOUS SOLUTIONS

3.1 Biosorption

Biosorption is a term that describes the broad range of processes by which biomass removes metals (and other substances) from solution, yet it can also be used in a stricter sense to describe uptake by dead (detritus) or living biomass by purely physico-chemical processes such as adsorption or ion exchange (White et al. 1995). Metabolic processes inherent in living biomass may contribute to the uptake mechanism. Ideally a biosorbent has the ability to be recycled and the sorbed metal ions recovered for reuse or safer disposal. Choice of a suitable biotrap is at times made easier if certain genetic and biochemical characteristics of an organism are known. That fungi and yeast can serve as biotraps for heavy metals has been the subject of a great deal of research as evidenced by several prior reviews on the subject (Blackwell et al. 1995; Kapoor and Viraraghavan 1995; 1997).

Predicting the effectiveness of a biotrap requires that some of its chemical composition be understood. For example, chitin and chitosan are well known metal-ion adsorbers due to the presence of both carboxyl and amine groups which make up these biopolymers (Ashkenazy et al. 1997; Cuero 1996; Fourest and Roux 1992; Juang et al. 1999). In fungi, the cell walls present a multilamellar architecture where up to 90% of the dry weight consists of amino- or nonamino-polysaccharides (Farkas 1980). The fungal cell wall is in essence a two-phase system consisting of a fibrous

chitin-based skeletal framework embedded within an amorphous polysaccharide matrix (Griffin 1994). Yeast and higher fungi such as the deuteromycetes *Trichoderma viride* have cell walls composed primarily of chitin and glucan polymers while the lower fungi such as *Rhizopus arrhizus* have cell walls of chitosan and chitin (Morley and Gadd 1995). Although chitosan does bind metal ions, the more highly polymerized and cross linked chitin performs better as a metal biotrap. However, Crusberg et al. (1994) reported that chitin, derived from a marine invertebrate source, was capable at best of binding 14.7 mg Cu²⁺/g biotrap at pH 4.0, with a binding constant of 27 mM, and concluded that this system was not likely to be developed commercially. There were better alternatives.

There is virtually no standardization in the literature for reporting details of metal-binding experiments with biotrap. Concentrations of metal ions are reported in several variations of mass per unit volume (mg/l, parts per million or ppm, and µg/l, parts per billion or ppb), as well as in moles per unit volume, millimolar (mmol/l) and micromolar (µmol/l). For example, copper ion (Cu²⁺) at 63 mg/l (ppm) may also be expressed as 1 mM but a uranium concentration of 1 mM would be almost four times larger in terms of mass per unit volume, at 238 mg/l. In fact, Cu²⁺ is often chosen as the model heavy metal in initial studies on a potential biosorbent because there are many quite sensitive analytical techniques available for its analysis. Most regulatory agencies mandate permissible metal ion concentrations in wastewater discharges in terms of mass per unit volume, e.g., ppm or ppb (parts per billion or µg/l). Other issues that should be properly addressed by investigators, but are often omitted in scientific reports, are effects of pH of an experiment on metal ion solubility, and ionic strength effects on equilibrium constants. For example, Cu²⁺ at 10 mg/l precipitates (as Cu(OH)₂) when the pH rises above 6.3, and precipitates at 1 mg/l when the pH rises above 6.8, based on a K_{sp} of 5 × 10⁻²⁰ (13*, 3*, 24). Ionic strength may affect rate constants which comprise an equilibrium constant according to Debey–Huckle theory. Even buffers used to maintain pH during uptake/binding studies should be chosen to minimize their chelating potential for the metal ions under consideration.

3.2 Heavy Metal Biosorption

Fungi and yeasts (Puranik et al. 1995; Sag and Kutsal 1996; Volesky and May-Phillips 1995; Zhao and Duncan 1998) have in the past received the most attention in connection with metal biosorption systems, particularly because plentiful amounts of fungal biomass are generated as by-products of several types of industrial alcohol and antibiotic fermentations (Omar et al. 1996; Sag et al. 2000; Volesky and Holan 1995). One such system consisting of *Aspergillus niger* waste from citric acid production was shown to remove zinc (as dust), magnetite, and metal sulfides from wastewater (Singleton et al. 1990; Wainwright et al. 1990). This process was shown to be independent of metabolism but was favored

by cell growth, with the particles eventually becoming entrapped within the matrix of the fungal hyphae. Al-Asheh and Duvnjak (1992) demonstrated that living mycelia of *Aspergillus carbonarius* were able to adsorb copper and chromium and noted that an increase in uptake correlated with an increase in pH. Sag et al. (1999) recently reported the simultaneous adsorption of chromate and divalent copper ions by living *R. arrhizus* in packed columns operated in continuous mode. Other fungal adsorbents were successfully demonstrated by Suhasini et al. (1999) to remove nickel ion from aqueous solutions, achieving uptake capacities of 214 mg Ni²⁺/g dry wt fungus.

Yeast similarly have proven to be effective research models for metal sorption studies. The abundant brewery and bakery yeast *Saccharomyces cerevisiae* has been the object of numerous biosorption studies. Wilhelmi and Duncan (1995) showed that immobilized cells of this yeast had the capability to adsorb Cu, Co, Cd, Ni, Zn, and Cr with an uptake averaging around 40 µmol/g in continuous flow packed bed columns through 8 repeated adsorption–desorption cycles. Optimum chromate removal from electroplating effluents has been recently demonstrated in fixed-bed columns at pH 2.5 using formaldehyde cross-linked *S. cerevisiae* (Zhao and Duncan 1998). Lead was found to adsorb at pH 5.5, to a nonliving cell mass of *Saccharomyces uvarum*, up to a maximum of 48.9 mg Pb/g dry wt biotrap. Given that carboxyl and amine groups served as the ligand to which the metal bound, it was assumed that chitin was the most likely provider of these groups (Ashkenazy et al. 1997).

Several authors now propose that biosorption processes utilizing whole cell biomass can realistically be considered as replacement technologies for existing metal-removal processes, or even as an effective polishing unit in place of existing treatment (Kapoor and Viraraghavan 1995; Volesky and Holan 1995). One recent promising report noted that dried powdered mycelium of *Fusarium flocciferum* can take up 19.2 mg Cd and 5.2 mg Ni, and between 4 and 6 for Cu, for each 100 mg of fungus (Delgado et al. 1998). Rather severe treatment regimens also may produce a biomass more capable of metal ion uptake. For example Brady et al. (1994) showed that hot alkali treatment of yeast biomass increased accumulation of divalent cation adsorption but limited success in removing chromate was reported. Formaldehyde treatment also was found to render baker's yeast more efficient in binding Cr(VI) (Zhao and Duncan 1998) but only a disappointing 6.3 mg Cr/g treated biomass was achieved. However Kapoor et al. (1999) recently reported that *A. niger* biomass was more effective than biomass treated by boiling in 0.1 N NaOH for the removal of Ni by biosorption. A number of questions regarding the behavior of dead vs. living biomass in the biosorption process was recently examined by Yetis et al. (2000) in a study of Pb(II) uptake by dead, resting and living *Phanerochaete chrysosporium* mycelia. Table 3 provides a comprehensive listing of a wide variety of biotrap (including some of bacterial origin as a comparison) capable of specifically sorbing copper from aqueous solutions.

Table 3 Copper biosorption by various types of microbial biomass

Organism Biomass type	Biomass class	Biosorption Capacity ^a (mg Cu/g)	Experimental operating conditions				Reference
			pH	T (°C)	C ^b (mg/l)	Bio-mass (g/l)	
<i>Z. ramigera</i>	Bacterium	270	5.5		0–500(e)	0.83	Norberg (1984)
<i>B. subtilis</i>	Bacterium	152					Brierley and Brierley (1993)
<i>Arthrobacter</i> sp.	Bacterium	148	3.5–6	30	180 (e)	0.4	Veglio et al. (1996)
<i>P. notatum</i>	Fungus	80					Siegel et al. (1980)
<i>C. tropicalis</i>	Yeast	80					Mattuschka and Straube (1993)
<i>Activated sludge bacteria</i>	Bacteria	50	5	25	15–200 (e)	0.5	Aksu et al. (1992)
<i>C. vulgaris</i>	Alga	42.9	4	25	10–260 (i)		Aksu et al. (1992)
<i>B. licheniformis</i> (CWP)	Bacterium	32	4	25	12–125 (i)		Beveridge (1986)
<i>Z. ramigera</i>	Bacterium	29		22	0–13 (i)	0.28	Cabral (1992)
<i>P. syringae</i>	Bacterium	25.4		25	1–320 (i)	1	Gadd and deRome (1988)
<i>C. resinae</i> (MP)	Fungus	25.4	5.5		5–50 (e)		Venkobachar (1990)
<i>G. lucidum</i>	Fungus	24	5		5–500(i)		dvan Say (2001)
<i>P. chrysosporium</i>	Fungus	20.2	6	25		1.05	deRome and Gadd (1987)
<i>R. arrhizus</i>	Fungus	19	5.5				Gadd et al. (1998)
<i>C. resinae</i>	Fungus	18					Volesky and May-Phillips (1995)
<i>S. cerevisiae</i>	Yeast	17	4–5	25	190 (e)	1	Tobin et al. (1984)
<i>R. arrhizus</i>	Fungus	16					Tobin et al. (1984)
<i>R. arrhizus</i>	Fungus	16		25	1–320 (i)	1	Gadd and deRome (1988)
<i>C. resinae</i>	Fungus	16	5.5				Huang et al. (1990)
<i>A. oryzae</i>	Fungus	13.6					Mattuschka and Straube (1993)
<i>P. guilliermondii</i>	Yeast	11					Mattuschka et al. (1993)
<i>S. cerevisiae</i>	Yeast	10					Mattuschka et al. (1993)
<i>S. obliquus</i>	Alga	10					Gadd et al. (1998)
<i>R. arrhizus</i>	Fungus	9.5	5.5	25	0.6–25 (i)		Niu et al. (1993)
<i>P. chrysogenum</i>	Fungus	9				3.5	Mattuschka and Straube (1993)
<i>S. noursei</i>	Bacterium	9	5.5	30	06–65 (i)	1	Gadd and deRome (1988)
<i>A. pullulans</i> (MP)	Fungus	9	5.5	25	1–320 (i)		Rao et al. (1993)
<i>A. niger</i>	Fungus	7.22					Brady and Duncan (1993)
<i>S. cerevisiae</i>	Yeast	6.3					Gadd and deRome (1988)
<i>A. pullulans</i>	Fungus	6	5.5	25	1–320 (i)	1	Mattuschka and Straube (1993)
<i>S. noursei</i>	Bacterium	5	5.5				Cotoras et al. (1993)
<i>Bacillus</i> sp.	Bacterium	5					Venkobachar (1990)
<i>A. niger</i>	Fungus	4	5		5–100 (e)		Townesley and Ross (1985)
<i>P. spinulosum</i>	Fungus	3.6					Galun et al. (1987)
<i>P. digitatum</i>	Fungus	3	5.5	25	10–50 (e)	6.5	Townesley et al. (1986)
<i>A. niger</i>	Fungus	1.7					
<i>T. viride</i>	Fungus	1.2					
<i>S. cerevisiae</i>	Yeast	0.8	4	25	3.2 (i)	2	Huang et al. (1990)
<i>S. cerevisiae</i>	Yeast	0.4	4	25	3.2 (i)	2	Huang et al. (1990)
<i>P. spinulosum</i>	Fungus	0.4–2					Townesley et al. (1986)

Table 3 is a compilation of data reported in previous reviews (Kapoor and Viraraghavan 1995; Veglio and Beolchini 1997; Volesky and Holan 1995). Areas in the table which are not filled imply that such data were not available to and/or not reviewed by the authors. References to the primary literature are given in the right-most column. CWP = cell wall preparation; MP = melanin preparation.

^a Metal uptake as reported is not necessarily at maximum.

^b (i) = initial concentration; (e) = equilibrium concentration.

3.3 Extracellular Precipitation of Metals

Exceeding a solubility product leads to precipitation of an insoluble salt of the reacting species. Sulfide anion (S^{2-}) and oxalate anion ($C_2O_4^{2-}$) are produced by some species of micro-organisms and these anions can form very insoluble salts with heavy metal ions which exhibit very small solubility products as noted by Veglio and Beolchini (1997). Copper phosphate precipitation occurring within the matrix of mycelia of the fungus *Penicillium ochro-chloron* after 4 days of incubation in shake flask cultures at pH 4, was demonstrated using scanning electron microscopy and energy dispersive x-ray microanalysis (Crusberg et al. 1994). Wrinkled 40–50 μ m dia. spheres of insoluble copper phosphate, inferred from EDX analysis, are trapped within the mycelia of the fungus grown for 4 days in aerated cultures in the presence of 100 mg/l Cu^{2+} . *Penicillium* and *Aspergillus* have been shown to produce extracellular acid phosphatases which correlate with copper removal from solution (Haas et al. 1991; Tsekova et al. 2002). It is likely that the extracellular phosphatases are localized in the periplasmic space, sandwiched between the membrane and cell wall materials. There they can act on extracellular organophosphates, hydrolyzing the phosphate and organic moieties for uptake via specialized transport systems. As a means of detoxification, the free phosphate anions can then react with and precipitate heavy metal ions as insoluble heavy metal phosphates before those ions enter the cell. *A. niger* has also been recently shown to immobilize metals as insoluble oxalate salts (Cunningham and Kuiack 1992; Sayer and Gadd 1997). In fact, oxalate is a common secondary metabolite of *Penicillium* spp. (Sayer and Gadd 1997) and is known to precipitate calcium oxalate under natural conditions in litter (Tait et al. 1999). Lead oxalate precipitation between pH 4 and 5 has also been demonstrated as a means of detoxification using living *A. niger* biomass (Sayer et al. 1999). Fungi therefore possess two mechanisms to protect themselves against extracellular heavy metals involving the precipitation of heavy metals as insoluble salts. Recently, Mukherjee et al. (2001) reported the bioreduction of $AuCl_4^-$ ions by *Verticillium* sp. In which gold nanoparticles 20 nm dia., characterized by a vivid purple color over the surface of the fungus growing on culture plates were observed.

3.4 Metal Biosorption by Specialist Isolated Molecules

Virtually all biological material has some affinity for toxic metals (Gadd and White 1993) and several mechanisms by which metals interact with microbial cell walls and envelopes are well established (Beveridge et al. 1997; Lovley et al. 1991). However some biomolecules function specifically to bind metals and are even genetically induced by their presence (Macaskie and Dean 1990). In *S. cerevisiae* the low molecular weight cysteine-rich polypeptide metallothionein

binds Cu^{+2} and Zn^{+2} as well as Zn^{+2} and provides resistance through detoxification of metal ions encountered in the natural environment (Turner and Robinson 1995).

3.5 The Special Case for Uranium

A serious effort has gone into finding biological means to reduce forms of more soluble uranium [U(VI)] compounds from aqueous waste streams, which appear at various stages of the nuclear fuel cycle. By exploiting the physiology of several different groups of microorganisms it has been shown that U(VI) can be adsorbed to microbial surfaces or biochemically converted to the very insoluble reduced [U(IV)] oxidation state, usually the uranite form (UO_2). Several organisms have been reported to carry out reduction of U(VI) to U(IV) anaerobically. A nonviable preparation of a thermotolerant ethanol producing strain of *Kluyveromyces marxianus* was found to rapidly take up uranium(VI) with an efficiency of up to 150 mg U/g dry wt biomass, but with lower binding at lower pH values (Bustard and McHale 1997; Bustard et al. 1997). Likewise, a cross-linked immobilized residual biomass from distillery spent wash was found capable of sorbing over 200 mg U/g dry wt biomass (Bustard and McHale 1997). A proprietary method using processed granules of the fungus *R. arrhizus* proved successful for uranium ion immobilization (Brierley and Brierley 1993) at 50 mg U/g biomass dry weight, in batch uptake studies. Complete uranium removal was demonstrated for dilute uranium ore bioleaching solutions (<300 mg/l) with eluate concentrations after desorption approaching 5000 mg U/l.

3.6 COMMERCIAL APPLICATIONS

Most living cell systems exploited to date have been used for decontamination of effluents containing metals at concentrations below toxic concentrations (Kapoor and Viraraghavan 1995; White et al. 1995). Some of those technologies employ a consortium of undefined micro-organisms as well as higher plants. The “Meander System” used at the Homestake lead mine (MO, USA) passes effluents containing Pb, Cu, Zn, Ni, Fe, and Cd ions through various ditches or channels containing autotrophic cyanobacteria, algae, and higher plants. Metal ion removal is achieved with an efficiency greater than 99% (Erlich and Brierley 1990; Jennet and Wixson 1983). These complex systems likely utilize precipitation and entrapment of particulates, in addition to biosorption and uptake into cells and plant tissues, in the removal process and concentrate the metals in the sediment in forms which greatly reduce environmental mobility and bioavailability (Brierley and Brierley 1993). In order to render sparse minerals in low grade ores soluble, those involved in biohydrometallurgy have exploited other micro-organisms to facilitate metal recovery (Brombacher et al. 1997) with the aim of mobilizing precious metals, Au, Ag, and sometimes Pt, but Ga, Ge. Patents (described in the

above references) outlining various processes employ reactor technology and the most simple heap-leach or in situ operations to strip metals from their ores. Bioleaching of metals from ores has successfully employed bacteria such as *Thiobacilli*, *Sulfobacilli*, and *Sulfolobus*, as well as consortia of organisms and some unidentified indigent species. The same practices, which are in use in the mining industry to liberate metals from ores perhaps have applications in the remediation of soils contaminated with those same metals by mobilization, followed by biosorption. Commercial processes using biosorbents for metal ion removal from aqueous waste streams are still in their infancy.

4 BIOREACTOR DESIGN

Bench-scale shake flask experiments rarely mimic the complexities encountered in unit operations where large volumes and short reaction times must be employed to ensure the economy of scale. Unit operations are preferred in the chemical industry since the process can usually be subject to computational modeling and thereby more predictable. Also, many industrial wastewaters contain several heavy metal contaminants and therefore competitive adsorption to sites on the surface of fungi has to be accounted for as described in a recent study involving *Penicillium chrysogenum* and *Streptovericillium cinnamomeum* by Puranik and Paknikar (1999). Biomass used in a reactor has to be either inexpensive and disposable or preferably in any case reusable after adsorbed metal ions are eluted. Yeast and fungi present somewhat difficult materials for carrying out column separation studies as the biomass often forms an impervious mat. However, the use of polyurethane foams as supports for biomass may help to overcome this problem (Dias et al. 2002; Tsekova and Ilieva 2001). Reaction times of biomass with metals to achieve sorption of metal ions must be short, yet many reports in the literature allow incubations to run for days to achieve suitable and reportable metal ion uptake. A typical experiment requires a 4-day incubation to prepare sufficient mycelia of *A. niger* for study and then another 24 hr for adsorption in shake flask following addition of metal ion (Price et al. 2001). However there are report of more rapid equilibration requiring from 10 to 30 min of contact (Puranik and Paknikar 1999). Immobilizing *A. niger* biomass within a polyurethane foam within an adsorption column increased copper uptake threefold compared with free mycelia studied in batch adsorption experiments (Tsekova and Ilieva 2001). Computational methods applied to unit operations permit the economy of a process to be predicted (Al-Asheh and Duvnjak 1992; Sag et al. 2001; Schiewer and Wong 1999). However, commercial scale bioreactors must address the problem of how a particular biotrap can render the influent metal concentration less harmful as the effluent is discharged. If it is to succeed, new biotrap technology must compete economically with those already at hand in the chemical industry. Sophisticated approaches have been reported for mathematical modeling of metal-biotrap sorption and precipitation

(Figueira et al. 1997; Schiewer and Volesky 1996; Veglio and Beolchini 1997). A mathematical Langmuir model has been recently developed for the prediction and performance of the simultaneous biosorption of Cr(VI) and Fe(III) by *R. arrhizus* in a semi-batch reactor. Likewise Sag et al. (2000) have recently reported the theoretical approach to and use of a continuous-flow stirred tank reactor for removing lead, nickel and copper from aqueous solution by nonliving *R. arrhizus* biomass.

5 LIMITATIONS AND POTENTIAL

The economic threshold for commercial selection of a biological process to replace a physico-chemical process for heavy metal removal from a waste stream as assessed by Macaskie (1991) is that a metal-loading capacity greater than 15% of the biomass dry wt must be demonstrated. Before the selection of any technology, it is imperative to note the hierarchy of hazardous waste management options: reduce; reuse; recycle.

The option of last resort is to treat and dispose of the waste in safe landfills, while minimizing the resultant volume, since disposal sites are few and space is precious not to mention expensive. A given bioremediation technology should be able to perform on a large scale in order for it to be commercially viable. The organism or biomaterial selected to accomplish the goal of removing or altering a heavy metal or metal ion rendering it less toxic must be very efficient in performing its intended function. The literature is rich with reports of studies attesting to the "potential" of a particular biomass or biomaterial to carry out bioremediation of metal-contaminated waste streams, but few have actually ventured beyond the laboratory bench scale. What is clear is the apparent dearth of genetic engineering reports in the literature. Classical genetic selection methods have proven useful, for e.g., for isolating a strain of *S. cerevisiae* out of 240 tested, capable of uptake of 3.2 mg chromium (Cr(VI)) per g dry wt of yeast cells (Liu et al. 2001). Genes which carry out certain functions in micro-organisms that provide them with resistance to heavy metals may be exploited for the development of new bioremediation technologies. Hunts for metal-resistant and metal ion-binding microorganisms appear to be very successful, but the investigators nearly always neglect to pursue the genetics behind their discovery. Silver recently reviewed the genetics of metal resistance by bacteria and noted several specific genes involved in metal uptake (Silver 1998). Many of these genes are located on plasmids and although not directly relevant to fungi and yeast they may have some characteristics in common with Eukaryotes. Ow (1996) has suggested that that employing *Schizosaccharomyces pombe* as a model system, and understanding of yeast metal tolerance genes may become clearer. Newly developed technologies can also be used to immobilize or encapsulate an isolated biotrap into gels where it can function in an *in vitro* manner if desirable. For example, gel-immobilized metal-binding proteins could then trap metal

ions in a more efficient manner and perhaps be amenable to the reverse reaction, enabling the recovery of the metal ion, and providing a reusable cost-effective system.

Recovery of heavy metals from contaminated soils and sediments by in situ bioremediation, however, remains elusive. To mobilize metals in those environments would likely threaten local groundwater, and when necessary the technology in current use, excavation and soil washing, may remain the only viable but expensive alternative. However, under certain conditions it may be possible to introduce metal-binding organisms or nonliving metal biotrap into soils and sediments to enhance the stability of metal ions at contaminated sites. Likewise, it may be possible to even enhance the population of indigenous metal-binding bacteria at contaminated sites by providing to them fixed carbon in gaseous form, e.g. methane or other volatile organics, essentially the reverse in situ bioremediation process known as bioparging. For the immediate future then, new heavy metal bioremediation technologies may be limited to the development of industrial applications for meeting regulatory agency requirements for pretreating wastewaters for later discharge.

6 CONCLUSIONS

Commercially feasible technologies involving traditional physico-chemical and chemical engineering principles are not at all simple or inexpensive, yet they are usually chosen over the few technologies using biological solutions currently available for heavy metal removal and recovery from industrial wastewaters. It is not to be expected that any bioremediation option will itself prove inexpensive. Perhaps it is not surprising that industry has been somewhat reluctant to adopt biological processes for use in metal-removal technologies. The chemical industry requires, and for good reason, reliable processes which are predictable, scalable, and controllable by plant operations personnel. Plant and laboratory aqueous effluents must consistently comply with discharge limits set by governmental regulators. As basic science reveals more and more about how biotrap behave, and as the number of potential materials reported in the scientific literature increases, many new opportunities for commercial adoption of a suitable biotechnology should begin to appear. Perhaps bioremediation of metal-contaminated sites and wastewaters will then be developed to its full potential and realize the promise of new and reliable technologies. The success will probably depend upon blending of the best technologies the chemical and biological fields have to offer.

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Decoloration of Industrial Wastes and Degradation of Dye Water

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1 INTRODUCTION

Large quantities of toxic and intensely colored waste effluents are produced by a range of industrial processes. These effluents are treated by processes ranging from aerobic to anaerobic and physico-chemical techniques. More innovative technologies using white-rot fungi have also been studied (Kahmark and Unwin 1996). The use of microorganisms to detoxify environmental pollutants is generally referred to as bioremediation. Environmental issues are now of increasing concern and biological methods are being directed towards technologies to minimize pollution or remedy it if it occurs.

White-rot fungi and their ligninolytic enzymes appear to have wide industrial potential. *Phanerochaete chrysosporium* and *Coriolus versicolor* can degrade lignin and a number of related chlorinated compounds. They are also able to efficiently decolorize and dechlorinate such effluents (Archibald et al. 1990; Fukui et al. 1992; Sundmann et al. 1981). Efficient color removal from sulfite pulping effluents has also been observed with *C. versicolor* (Bergbauer et al. 1991; Royer et al. 1983). Furthermore, white-rot fungi are showing significant potential to biodegrade a large number of xenobiotic compounds and hazardous wastes (Field et al. 1993; Glaser 1990; Hammel 1989). Lignin peroxidase, manganese dependent peroxidase, and laccase are the three main ligninolytic enzymes (Leisola and Garcia 1989; Schoemaker et al. 1989) and have been well-studied but several other enzymes are involved in further degradation of the products resulting from their action.

This chapter describes the rationale and results for three related case studies using a white-rot fungus to (a) treat effluent wastewater to reduce color, (b) to decolorize dye solutions used in industrial processes, and (c) to determine the enzyme involved in the decolorization process.

2 ORGANISMS DEGRADING LIGNIN

Lignin is an amorphous, highly aromatic heterogenous, 3-D branched polymer (Buswell and Odier 1987; Higuchi 1985) with its substituents connected by carbon-carbon and ether linkages. There are different lignin structures consisting of various amounts of the three cinnamyl alcohol precursors present: *p*-coumaryl, coniferyl, and synapyl alcohols. These alcohols give rise to *p*-hydroxyphenyl units, guaiacyl units, and syringyl units, respectively, in lignin (Higuchi 1985; Kirk and Farrell 1987). The lignin cinnamyl alcohol precursors produce phenoxy radicals which randomly couple.

White-rot is characterized by the bleached white color of advanced wood decay due to fungi (white-rot fungi) and other organisms. White-rot can totally degrade all major wood polymers, including lignin (Crawford and Crawford 1980; Kirk 1971). Lignin is significantly de-aromatized. The degradation follows two general patterns (a) simultaneous attack on lignin and polysaccharides or (b) preferential or selective removal of lignin and/or hemicellulose without extensive depletion of cellulose (Buswell 1991). White-rot fungi degrading lignin in preference to polysaccharides are *Pycnoporus cinnabarinus*, *Pleurotus ostreatus*, *Phlebia radiata*, and *Phlebia (Merulius) tremellosus* (Ander and Eriksson 1977).

Other organisms degrading lignin include the brown-rot fungi, the soft-rot fungi, which seem capable of some demethylation and possibly degradation of side chains and aromatic rings (Ander and Eriksson 1978; Buswell 1991) and some bacteria associated with the Actinomycetes and Eubacteria (Kirk 1971). Bacteria may extensively degrade, demethoxylate, and modify aromatic rings and side chains.

3 DECOLORIZATION OF DYES BY MICROORGANISMS

Synthetic azo, anthraquinone, triarylmethane, and other dyes are extensively used for textile dyeing, paper printing, and color photography. A necessary criterion for the application of these dyes is that they resist photodegradation and washing processes. In addition, these dyes are also resistant to microbial degradation and are not easily removed from wastes during conventional biological treatment (Meyer 1981; Shaul et al. 1991). The removal of synthetic dyes from wastewaters is mainly achieved by elimination of the dye stuffs using adsorption, chemical oxidation, and flocculation.

Azo linkages and aromatic sulfo groups do not occur naturally and they resist oxidative degradation. Partial degradation has been observed for anthraquinone and azo dyes during aerobic activated sludge treatment, but substantial removal of color from the wastewater was attributed to adsorption of the intact dye onto the sludge (Athanasopoulos 1991). The dyestuffs are generally more susceptible to anaerobic sludge degradation. The anaerobic reduction of azo dyes to colorless products by bacteria has been reported (Meyer 1981). The initial step in this transformation is the reductive fission of the azo group, resulting in the formation and accumulation of toxic and possibly carcinogenic aromatic amines. The rapid decolorization of a number of textile dyes and effluents by an immobilized anaerobic consortium had also been demonstrated (Nigam et al. 1996; Oxspring et al. 1996).

The ability of *P. chrysosporium* to decolorize polymeric dyes, such as Poly B-411, Poly R-481, and Poly Y-606, has been correlated with its lignin degrading system (Glenn and Gold 1983; Gold et al. 1983; 1988). Strains of *P. ostreatus* have also been shown to effectively decolorize Poly B-411 (Platt et al. 1985). *P. pulmonarius* increased Poly B decolorization, as well as enhanced laccase production. Laccase is often produced by *Pleurotus* sp. as part of their ligninolytic system but dye decolorization by this fungus

appears to be unrelated due to increased laccase secretion (Masaphy and Levanon 1992).

The utilization of the white-rot fungi and their nonspecific ligninolytic enzyme system for the biodegradation of other industrial dyes has found increased interest, since it has also become known that *P. chrysosporium* is able to degrade and decolorize a range of azo, sulfonated dyes, heterocyclic, and triphenylmethane dyes (Bumpus and Brock 1988; Cripps et al. 1990; Paszczynski et al. 1992; Spadaro et al. 1992) (Table 1). The triphenylmethane dye, crystal violet, has been decolorized by *P. chrysosporium*. Three metabolites were identified and the dye was N-demethylated by purified lignin peroxidase providing further evidence that the ligninolytic system is at least involved in oxidative biodegradation (Bumpus and Brock 1988). Pararosaniline and cresol violet and cresol red were also decolorized.

A number of other azo, sulfonated azo, triphenylmethane, and heterocyclic dyes can also be degraded by various strains of *P. chrysosporium* (Table 1). This further demonstrates the usefulness of the ligninolytic system in biodegrading and decolorizing wastewaters contaminated with dyes from textile and other industries (Cripps et al. 1990). Fluorescein acid (Gogna et al. 1992) and thiazine dyes (Kling and Neto 1991) have also been degraded by *P. chrysosporium* or lignin peroxidase. Many studies have used *P. chrysosporium*, although a H_2O_2 -dependent enzymatic decolorization by *P. ostreatus* has also been shown (Vyas and Molitoris 1995). Knapp et al. (1995) suggests that more work on the effect of the decolorizing capacity of other members of the Basidiomycetes is warranted.

4 LIGNINOLYSIS AND DECOLORIZATION BY ENZYMES

A number of white-rot fungi and/or their enzymes have been used successfully in detoxification and degradation of

Table 1 Dyes decolorized by *Phanerochaete chrysosporium*

Organism	Dye class	Dye	References
<i>P. chrysosporium</i> BKM 1667 (ATCC 24725)	Sulfonated azo dyes	Synthesized dyes	Paszczynski et al. (1992)
<i>P. chrysosporium</i> OGC 101	Azo dyes	Disperse yellow 3	Spadaro et al. (1993)
		Disperse orange 3	
		Solvent yellow 14	
<i>P. chrysosporium</i> BKM-F-1767	Azo dyes	Tropabolin O	Cripps et al. (1990)
		Orange II	
		Congo red	
	Heterocyclic dyes	Azure B	
<i>P. chrysosporium</i> ME-446	Polymeric dyes	Poly B-441	Glenn and Gold (1983)
		Poly R-481	
		Poly Y-606	
<i>P. chrysosporium</i> BKM-F-1767	Triphenylmethane dyes	Crystal violet	Bumpus and Brock (1988)
<i>P. chrysosporium</i> BKM-1667 (ATTC 24725)	Fluorescein acid dye	Rose Bengal	Gogna et al. (1992)

selected xenobiotics. The most studied white-rot fungus is *P. chrysosporium*.

Most of the oxidative reactions observed are presumed to be one-electron oxidation of suitable phenolic substrates to yield phenoxy radicals. Nonphenolic substrates yield radical cationic intermediates (Kirk and Farrell 1987). The result is the formation of opened-ring products, small aromatic fragments, and quinones and these can be further metabolized.

4.1 Lignin Peroxidase

A lignin-degrading enzyme from *P. chrysosporium* was identified in 1983 by two independent research teams (Glenn et al. 1983; Tien and Kirk 1983). This enzyme was termed ligninase, but it is now generally known as lignin peroxidase. The enzyme was shown to partly depolymerize lignin, but can cleave C α –C β linkages in side chains suggesting that the depolymerizing and ring cleavage ability of this enzyme constitutes its main function in lignin degradation (Chen and Chang 1985; Eriksson et al. 1990).

The enzyme has also been found in other fungi including *C. versicolor* (Dodson et al. 1987; Jönsson et al. 1987; Waldner et al. 1988), *P. radiata* (Hatakka et al. 1987; Kantelinen et al. 1988; Niku-Paavola 1987; Niku-Paavola et al. 1988) and *P. tremellosus* (Biswas-Hawkes et al. 1987).

4.2 Manganese Dependent Peroxidase

One year after detection of lignin peroxidase, Kuwahara et al. (1984) reported the isolation of another enzyme fraction from *P. chrysosporium* strain ME 446. This fraction showed Mn(II), H₂O₂ and lactate dependency and stimulation by increased protein concentration in reaction mixtures. The enzyme can oxidize a variety of dyes, including phenol red, *o*-dianisidine, and Poly R. Purification and characterization of this manganese dependent peroxidase was subsequently carried out (Glenn and Gold 1985).

A similar enzyme, tentatively named vanillylacetone peroxidase was purified from *P. chrysosporium* strain BKM-1767 (Paszczynski et al. 1985). This enzyme oxidized various low-molecular weight phenols and an aromatic amine in the presence of Mn(II) and H₂O₂. It did not oxidize phenol red and was not activated by lactate. The enzyme oxidized NADPH and reduced glutathione (GSH) thereby, possibly, indicating a link with xenobiotic metabolism. Hydrogen peroxide was formed in this reaction. Generation of hydrogen peroxide from GSH occurs with a manganese dependent peroxidase from *Lentinus edodes* (Forrester et al. 1988).

Manganese accumulation in the decayed residue of wood (Blanchette 1984) supported the hypothesis that manganese dependent peroxidases participated in lignin degradation (Paszczynski et al. 1985) via oxidation to a higher oxidation state (Glenn et al. 1986; Paszczynski et al. 1986). Manganese

dependent peroxidase oxidation of Mn(II) to Mn(III) was subsequently demonstrated (Glenn et al. 1986).

4.3 Laccase: Role and Function in Lignin Biodegradation

Laccases classified as polyphenoloxidases are now widely accepted as *p*-diphenoloxidases (EC 1.10.3.2) (Tuor et al. 1995) and are widespread. The enzyme is found in many plant species (Mayer and Harel 1979) and is widely distributed in fungi (Mayer 1987) including wood-rotting fungi where it is often associated with lignin peroxidase, manganese dependent peroxidase, or both (Nerud and Mišurcová 1996; Tuor et al. 1995). They are remarkably nonspecific as to reducing substrates and show much in common with another copper-containing oxidase, tyrosinase (monophenol mono-oxygenase; EC 1.14.18.1).

Laccases are blue copper oxidases and participate in electron transfer in biological systems by catalyzing the four-electron reduction of dioxygen to water with the simultaneous oxidation of organic substrates (Reinhammar and Malmström 1981; Thurston 1994). A minimum of four copper atoms, distributed in three spectroscopically distinct binding sites, appears necessary to facilitate efficient catalysis. The Type I (blue) copper center has a strong absorbance near 600 nm and gives rise to the enzyme's blue color. The other two copper centers known as the Type II (normal) and the Type III involve a pair of magnetically coupled cupric ions (Farver and Pecht 1981; Li et al. 1992; Morpurgo et al. 1993). Studies into the mechanism of the reduction of O₂ to H₂O by laccases have shown that a bridge between the coupled binuclear center and the Type II center defines a trinuclear cluster as the active site (Solomon 1988).

Plant laccase mechanism and function has been investigated predominantly on *Rhus vernicifera*. It is thought that the enzyme has a protective function, possibly causing formation of a natural polyphenolic polymerisate in the case of tree damage (Reinhammar and Malmström 1981). A plant laccase has also been found to be involved in lignification (Bao et al. 1993) and the role of laccase in lignification has been reviewed extensively by O'Malley et al. (1993).

Fungal laccases oxidize phenols and phenolic substructures of lignin with subsequent polymerization or depolymerization (Higuchi 1989). These pathways are considered to proceed via phenoxy radicals of the phenolic units. The role of laccase in lignin biodegradation remains largely unresolved, since strains of the extensively studied white-rot fungus, *P. chrysosporium*, and several other Basidiomycetes are active in lignin degradation but do not produce laccase under the ligninolytic conditions employed in laboratory studies (Nerud and Mišurcová 1996; Thurston 1994; Tuor et al. 1995).

In some fungi, laccase has a function seemingly unrelated to ligninolysis. For example, in *Aspergillus nidulans*, the

enzyme appears to be essential for the synthesis of the spore pigments (Clutterbuck 1972).

5 CASE 1—DECOLORIZATION OF AN INDUSTRIAL WASTE

5.1 The Composition of the Waste

The initial problem was to decolorize the complex wastewater from an industrial site. The waste originated in a pigment plant and advice indicated that it contained low molecular weight alkyd (sic) polyester resins derived from the reaction of triglycerides and anhydrides like phthalic anhydride along with epoxide resins, derived from bisphenol, and epichlorohydrin and amine resins from melamine and formaldehyde. Lesser quantities of higher molecular weight materials, from polymerization of vinyl, acrylate, or methacrylate monomers were also present. The effluent had a total organic carbon content (TOC) of 2770 mg/l and a total suspended content (TSC) of 7429 mg/l.

5.2 Selection of Decolorizing Organisms

In view of the numerous reports of involvement in dye decolorization and lignin degradation (Sections 2 and 3) the white-rot fungi were considered the organisms of choice for this work. It was essential that the organism could either grow on or metabolize the effluent. Plates of malt extract agar (MEA) were prepared in the effluent diluted to contain 695, 1389, 2083, and 2770 mg/l TOC, respectively. They were adjusted to pH 4.5 to aid fungal growth. Plates were centrally inoculated with several white-rot fungi that had been collected in Victoria, Australia, and some standard strains. The plates were grown under high humidity and examined daily.

The most appropriate organism was judged to be a strain of a Victoria isolate of *P. cinnabarinus*, identified according to Fuhrer (1985) and Macdonald and Westerman (1979). In the presence of a primary carbon source (and a TOC of the effluent of 1385 mg/l) this fungus covered the entire plate in 96 h. This TOC concentration was also used in bioreactor experiments.

5.3 Conditions in a Packed-Bed Bioreactor

Most degradation studies employing *Phanerochaete* have been carried out in solid cultures or in shallow liquid stationary or shake cultures (Ander and Eriksson 1977; Hatakka 1985). Packed-bed bioreactor configurations have been mainly used to study the ligninolytic enzymes and degradative abilities of *P. chrysosporium* (Lewandowski et al. 1990; Linko 1988). In view of this it was decided to examine the effect of the fungus on the industrial waste in a 2 l packed-bed reactor.

The strain of *P. cinnabarinus* was inoculated onto a nylon web cube support (0.5 cm³ of Scotchbrite®). The effluent was prepared in a defined medium (Tien and Kirk 1988) which was modified by increasing the final concentration of diammonium tartrate to 0.8 g/l to give a carbon to nitrogen ratio of 38.3:1. Although it stimulated phenoloxidase activity, veratryl alcohol (40 mM) was omitted in this trial because it retarded mycelial growth, inhibited glucose and nitrogen utilization and delayed decolorization. Addition of veratryl alcohol at the time of inoculation may interfere with fungal metabolism (Tonon and Odier 1988).

The medium was circulated through the reactor at 0.45 l/h and samples were taken daily and analyzed for glucose concentration, available nitrogen, and oxidative enzyme activity which at this stage was termed phenoloxidase. A control reactor lacking the effluent was also used.

5.4 Decolorization in the Reactor

The results are summarized as follows for a 15-day reactor cycle. The desirable characteristics of vigorous growth and rapid decolorization (Joyce et al. 1984) were achieved by omitting veratryl alcohol from the medium in the reactor cycle. In this case, the nitrogen was consumed in 24 h and glucose in 48 h. The phenoloxidase reached a level of 4.8 units of ABTS* oxidized/ml on day 9 and then declined to remain at a relatively steady level of 1.85 U/ml for the rest of the cycle. This decrease broadly coincided with detection of autolytic activity and reappearance of nitrogen in the medium. The dark effluent was rapidly decolorized during the first 24 h. The fungal mycelium was originally dark suggesting the adsorption of some of the solid by the mycelium. This intensity decreased over the 15-day cycle until the mycelium became indistinguishable from the control. The final effluent was a light yellow.

There were differences in the spectra at different stages of decolorization of the effluent and some of these are shown in Figure 1. In this increased decolorizing effect three stages are suggested. Firstly some of the color adsorbs to the enhanced mycelial mass formed in the absence of veratryl alcohol. Secondly, the phenoloxidase acts to decolorize the soluble dye components of the effluent and thirdly the phenoloxidase acts on the adsorbed color of the mycelium *in situ*. Evidence that the phenoloxidase was most probably a laccase is reported in Section 6.

6 CASE 2—DECOLORIZATION OF CHICAGO SKY BLUE

6.1 Structure of the Dye

Chicago Sky Blue (Figure 2) is a disazo dye used extensively in dyeing cotton and cellulosic fiber material. It is often

*One unit (U) of enzyme activity is defined as the amount of enzyme causing a change in absorbance of 1.0 min⁻¹ at room temperature (Bourbonnais and Paice 1990).

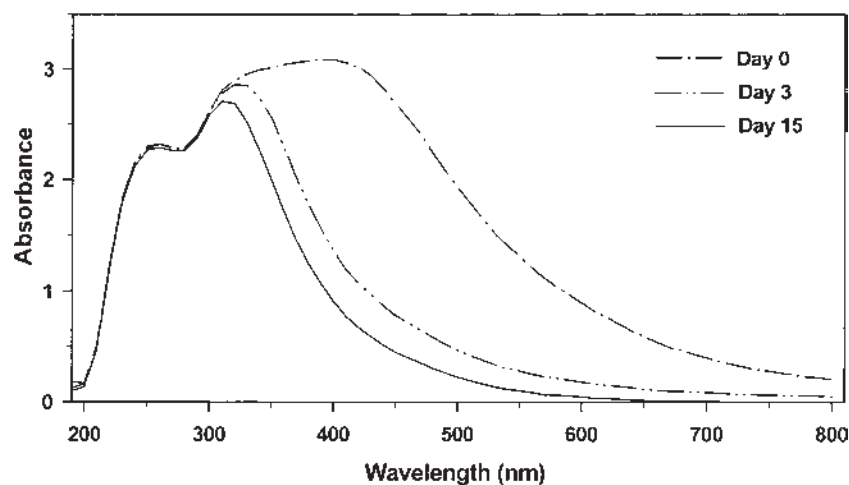


Figure 1 Decolorization of effluent by *P. cinnabarinus*.

incorporated into inks, and used for dyeing of leather and paper. It belongs to the largest class of commercially produced water-soluble dyes that are characterized by up to three azo linkages which link phenyl and naphthyl groups containing combinations of functional groups (sulfonic acid, sodium salt; amino; chloro; hydroxyl; methyl and nitro) on the rings.

Decolorization and degradation of azo dyes have been demonstrated with *P. chrysosporium* (Cripps et al. 1990; Ollikka et al. 1993; Pasti-Grigsby et al. 1992; Paszczynski et al. 1991; 1992; Spadaro et al. 1992). A lignin peroxidase of this organism catalyses the decolorization of azo, heterocyclic, triphenylmethane, and polymeric dyes (Ollikka et al. 1993).

Much less is known about the role of laccases in the decolorization of synthetic dyes, although several wood-rotting fungi produce laccase as well as peroxidases and have been shown to decolorize a range of structurally different dyes (Knapp et al. 1995). A mechanism for degradation of phenolic azo dyes by laccase from *Pyricularia oryzae* (Chivukula and Renganathan 1995) is similar to the one proposed for degradation of phenolic azo dyes by a lignin peroxidase (Chivukula et al. 1995). In the latter case formation of novel sulfophenyl hydroperoxides was postulated and the elimination of the azo linkage as molecular nitrogen. However, in the case of laccase, electron-rich

phenolic rings adjacent to the azo linkage appeared necessary for oxidation and phenoxy radical formation. Mass spectral (MS) and electrospray ionization (EI)-MS analyses confirmed the release of azo linkages as molecular nitrogen. This prevented aromatic amine formation.

6.2 Decolorization of CSB Using *Pycnoporus cinnabarinus*

The isolate of *P. cinnabarinus* was examined for its ability to decolorize CBB on plates initially, then in shake flasks and subsequently in 2 and 10l packed-bed bioreactors. In every case the dye was decolorized at relatively high concentrations (500 mg/l).

6.3 Enzymes Involved in the Degradation

The decolorization of the dye by *P. cinnabarinus* led to a search for the responsible enzymes. In the early experiments, solutions had been tested in the presence of a catalase from *Aspergillus niger*. Despite the catalase the ABTS was still oxidized. This suggested an involvement of laccase in the decolorization reaction and a search was undertaken to see whether it acted alone or whether it required other mediators.

6.4 Production and Purification of Laccase from *Pycnoporus cinnabarinus*

In the 10l packed-bed bioreactor, *P. cinnabarinus* used glucose at a constant rate. The ammonia content of the medium was exhausted by day 4. Laccase activity was first detected on day 4 and had reached 0.28 U/ml by day 10 when the culture broth was harvested for laccase purification.

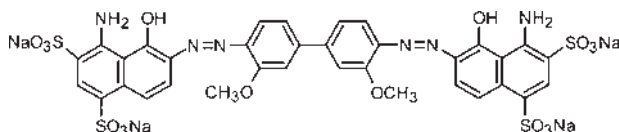


Figure 2 Structural formula of Chicago sky blue (CSB).

The isolation procedure follows that of Schliephake et al. (2000). Laccase was purified from 7.9 l of culture fluid in two ultrafiltration steps followed by concentration, dialysis, and column chromatography on Sephadex G 75. Fractions absorbing at 614 nm were analyzed for laccases activity, pooled, dialyzed against 20 mM histidine buffer pH 6, and again concentrated. The concentrate was chromatographed on a 5/5 Mono Q anion exchange column with a sodium chloride gradient of 0–0.5 M over 50 min at 1 ml/min. Fractions absorbing at 614 nm were pooled and stored at -85°C in the presence of a protease inhibitor mixture. A 45-fold increase in specific activity was obtained on purification. Capillary electrophoresis estimated purity at greater than 95% with minor peaks (areas 0.079 and 4.596%) at longer elution times.

6.5 Characterization of Laccase

The enzyme contained 510 amino acid residues and was a single polypeptide of a molecular size of 63 kDa. It contained 11% carbohydrate that is consistent with other laccases (Coll et al. 1993). The first 10 amino acids at the N-terminal were A, I, G, P, V, A, B, L, T, and L and differed from the enzyme from *P. cinnabarinus* PB, also isolated in Australia, only in the B for D in residue 7 (Eggert et al. 1996). It also showed considerable homology to the laccase of *P. ostreatus* (Youn et al. 1995), *C. (Trametes) versicolor* (Bourbonnais et al. 1995), and *Coriolus hirsutus* (Kojima et al. 1990).

A major band and two minor bands were observed at pH 3 and pH 2.85 and 2.65, respectively, on isoelectric focusing (IEF). Each band stained the laccase substrate guaiacol (10 mM). One band only, corresponding to laccase activity, was obtained on electrophoresis (4–20% gradient gel) under nondenaturing conditions. This band, stained with 10 mM guaiacol, corresponded with the co-electrophoresis protein band.

6.6 Properties

The ultra-violet visible (UV/Vis) absorption spectrum of laccase was similar to that observed of other fungal laccases (Coll et al. 1993; Eggert et al. 1996) with a Type I band at 614 nm, corresponding to the blue copper of laccases (Reinhammer and Malström 1981) and a broad band at 330 nm indicative of a Type III binuclear copper center (Malkin et al. 1969; Reinhammer 1984). The EPR spectrum of purified laccase had two superimposed signals of Type I and Type II copper centers.

The laccase was stable at 60°C for 1 h and still retained much activity after 2 h incubation at 80°C . It differed from the laccase of *P. cinnabarinus* PB (Eggert et al. 1996) which was inactivated after incubation at 80°C for 1 h. The enzyme was still active in bioreactors run at 37°C for 25 days but lost activity on prolonged treatment at the higher temperatures.

The pH optimum for syringaldazine was between pH 4.4 and 5 and for guaiacol between pH 4 and 4.5. The K_m was

0.03 mM for syringaldazine and 0.33 mM guaiacol. Activity was irreversibly inhibited by 0.1 mM sodium azide and by ascorbic acid at 1 mM.

6.7 Decolorization Using Purified Enzyme

In degradation studies, the absorbance of the dye at 618 nm (25 mg CSB/l) was monitored against a heat treated blank. The rate of decolorization was initially linear (0.82–25 μg protein), and proportional to enzyme concentration but equilibrium was reached after 2 h. The major CSB peak at 618 nm and minor peak at 320 nm disappeared and a new peak appeared at 550 nm.

The dye had a retention time of 22 min on HPLC (C_{18} reversed phase column) (Schliephake et al. 2000). After 20 h reaction time the 22 min peak disappeared and two new peaks appeared with retention times of 1.28 and 3.96 min, respectively. Their molar absorptivities were an order of magnitude lower than CSB. Thus, the disazo dye CSB had been clearly degraded by the activity of the laccase. The products have not been characterized so far.

7 CASE 3—DECOLORIZATION OF REMAZOL BRILLIANT BLUE

7.1 Laccase and Remazol Brilliant Blue

Laccase was shown to be a major extracellular enzyme of *P. cinnabarinus* and responsible for decolorization of CSB and most likely the pigmented wastewater. A number of successful trials of the strain *P. cinnabarinus* in decolorizing the vinyl sulfonyl industrial dye, Remazol Brilliant Blue R (RBBR) (Figure 3), were carried out on the laboratory scale (Jones et al. 1993; Lonergan et al. 1993). Remazol Brilliant Blue R is used for dyeing jeans. Subsequently a purified laccase from *P. cinnabarinus* was also shown to be involved in the decolorization of industrial grade and purified RBBR (Lonergan et al. 1996).

It therefore seemed appropriate to monitor the laccase development of *P. cinnabarinus* during a dye decolorization reactor cycle on a pilot scale prior to an industrial scale up. A 200 l packed-bed bioreactor was designed for the pilot scale run (Lonergan et al. 1995a,b). This pilot reactor trial provided much larger and more active samples from which laccase could be purified.

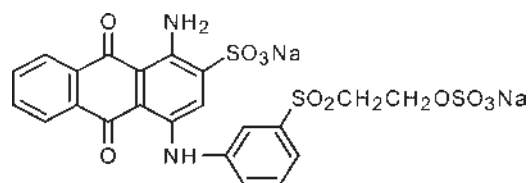


Figure 3 Structural formula of RBBR.

7.2 The Pilot Scale Bioreactor

The fermentation was conducted in a 200 l aerated polythene drum, packed with 2 cm³ nylon (Scotchbrite®) cubes (Lonergan et al. 1995 b). A reactor cycle of 25 days was used in this instance with the medium continually recycled at $36 \pm 1^\circ\text{C}$. *P. cinnabarinus* rapidly colonized the packing. Veratryl alcohol (4 mM) was used as a laccase inducing agent. The RBBR was added (500 mg/l) to the medium on day 3 after inoculation. Daily samples were analyzed for glucose, nitrogen, and phenoloxidase (laccase) activity. A sample was also taken 30 min after addition of the dye and is termed D3 + 30.

7.3 Decolorization of Dye in Bioreactor

The dye (595 nm) was decolorized by 80% within 24 h and almost completely decolorized within 48 h after addition. The nitrogen in the reactor was exhausted in 7 days and the glucose exhausted in 12 days whereupon more glucose was added to the reactor (Lonergan et al. 1995b). There was a small increase in nitrogen towards the end of the cycle due to autolysis.

Laccase activity was detected on day 2, peaked at a very large 60 U/ml on day 8 and fell to a minimum on day 12. It then rose again towards the end of the cycle as the additional glucose was added.

In this series of experiments laccases were purified from 100 ml samples removed during the reactor operation on day 3, D3 + 30, 4, 20, 23, and 25 by a slight modification to the method reported (Schliephake et al. 2000). The elution profiles for all samples were similar to those of the laccase purification carried out on the Mono Q anion exchanger. The elution pattern for days 4 and 23 are shown in Figure 4. The anomaly in enzyme elution times (day 4, 18.5 min and day 23, 15.5 min) was examined.

7.4 Variation in Enzyme

The apparent molecular weight of laccase, previously estimated at 63 kDa by SDS-PAGE (Schliephake et al. 2000) was consistent in the laccase from days 3, D3 + 30 min, 4, and 20. The protein bands from samples on days 23 and 25 migrated further in the gel and gave a molecular weight of 54,620.

On electrophoresis of the purified reactor samples, under native conditions, and visualizing laccase active bands with guaiacol, significant variation occurred in the migration of the enzyme through the gel from different days. Electrophoretic migration of proteins under nondissociating buffer conditions occurs on the basis of both size and charge (Hames 1990). This variation in migration indicated that physical transformation of the enzyme may have occurred during the reactor cycle without loss of the enzyme activity.

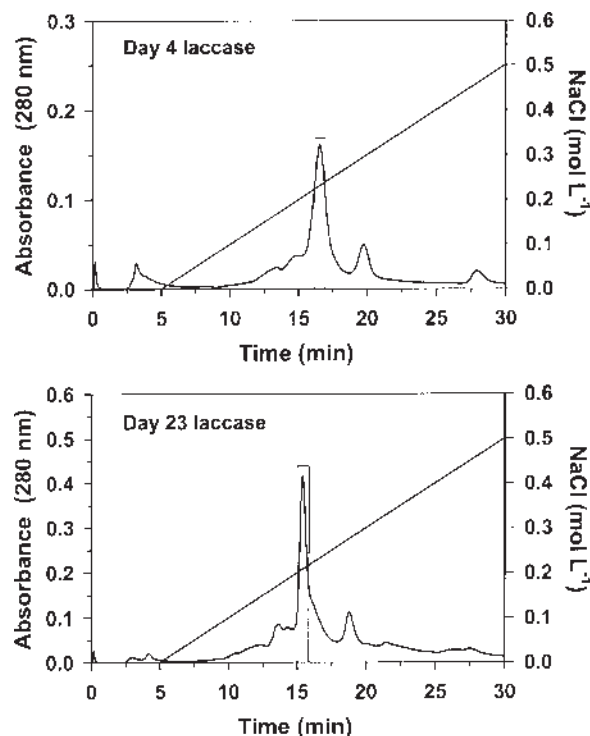


Figure 4 Elution profile of laccases from day 4 (A) and day 23 (B). The 100 ml sample was concentrated to 5 ml, passed through Sephadex G 75 and active fragments pooled. The active solution was concentrated through Centricon ultrafilters and the concentrate passed through a Bioscale 5 anion exchange column, using the same gradient as for the Mono Q column but spread over 35 min and at a same flow rate of 1 ml/min.

Purified laccases were subjected to IEF and stained for activity. The enzymic banding pattern showed significant variation on days 3, 4, 20, 23, and 25. The activity stain showed that on day 3, prior to the addition of the dye to the reactor, five bands stained for laccase activity, with the strongest staining bands towards the anode. On day 4, six bands were observed and the band closer to the cathode had the stronger stain. A similar pattern was also observed on day 20 of the reactor cycle. On day 23 and 25, however, there were only four bands and the band nearest the anode was again the strongest staining band. Such a pattern of laccase on IEF was considered artifacts due to the nature of the gel (Eggert et al. 1996). The variations, together with the migration on the native PAGE, however, did not exclude modification of the enzyme over the reactor cycle.

The decolorizing activity of laccases during a 7 h incubation varied substantially for the different days investigated (Figure 5). Day 4 laccase decolorized RBBR at the fastest rate. This particular enzyme had been isolated from the reactor one day after the addition of the dye. The second most active laccase was isolated from day 3, i.e., before dye

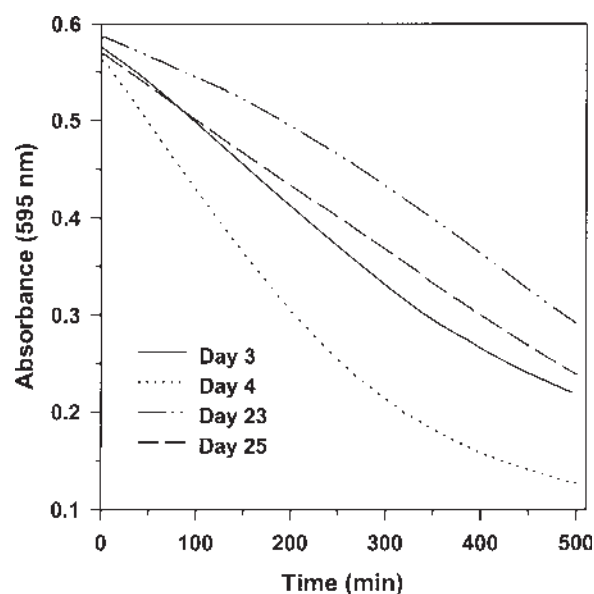


Figure 5 Decolorization of dye RBBR by laccases from various days. Laccase preparations (15 μ g protein) from day 3, 4, 23, and 25 were mixed with 1 ml of RBBR solution (50 mg/l) and the absorbance at 595 nm monitored for 7 h.

addition. Laccases from days 23 and 25 were initially less efficient. However, the spectra after 20 h reaction showed the extent of removal of the peak around 595 nm and the most efficient color removal was observed for laccases from day 4 and 25 (Figure 6). Although the rate of decolorization for day 25 laccase had appeared slower over the first 7 h (Figure 5), its decolorization efficiency had proceeded further after 20 h. This observation is probably related to the observed differences in electrophoretic properties.

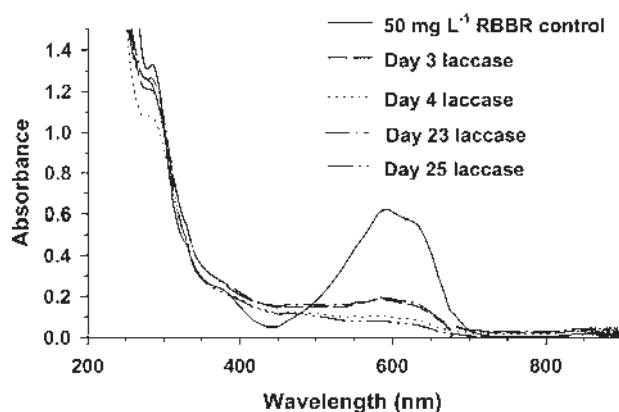


Figure 6 UV/Vis spectra of solutions shown in Figure 5 after a 20 h reaction time.

8 CONCLUSIONS

A strain of *P. cinnabarinus* was isolated which decolorized an industrial waste as well as the disazo dye CSB and the vinyl sulfonyl dye RBBR. On purification the phenoloxidase of the enzyme showed all the characteristics of a laccase. The purified laccase from the white-rot fungus rapidly decolorized both dyes. There appeared to be some charge to size variations of laccase during the reactor cycle. It may be hypothesized that the dye may have acted on a proportion of laccase molecules in such a way that it modulated the protein. This could give rise to a conformational change, which may optimize the catalytic site of the enzyme in such a way that it is more easily accessible. As the dye molecule is very small (FW $\sim$ 625) and contains a free aromatic amino group, there could be a possible reaction occurring between a possibly free carboxyl groups of an exposed amino acid of laccase and the amino group of the dye. This would mean that such amino-containing molecules under certain circumstances might behave like amino acids. Such a modulation could possibly explain the presence of an additional acidic band appearing closer to the cathode upon exposure to the dye.

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Bioconversion of Distillery Waste

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1 INTRODUCTION

Alcoholic beverages containing higher concentrations of alcohol than can be obtained by wine fermentation have to be prepared by distillation. After the distillation process all the ingredients of the original fermented mash or liquid, except volatiles, remain in the waste, called stillage, spent wash, slop, or vinasse. The composition of this distillery waste depends on the original raw materials used: sugarcane- or sugar beet molasses, corn, wheat, barley, cassava, potato, rice, fruits, etc. These materials have to be prepared for alcoholic fermentation by dilution or mashing, including eventual hydrolysis and/or addition of nutrients. Stillages remaining after distilling off the alcohol are troublesome wastewaters that are either pulpy materials or clear fluids containing settling particles. The stillages are about 10–14 times the volume of the alcohol produced and have a high content of organic substances (Shojaosadati et al. 1999). The pollution load is quantified by biochemical oxygen demand (BOD) and chemical oxygen demand (COD). Distillery stillages are characterized by a very high BOD, very high COD, and a low pH value. In addition, with certain types of distillery wastewater such as fruit slops, the separation of solids from the liquid is almost impossible (Friedrich et al. 1986). There are substantial pressures to develop a proper treatment for making wastes acceptable. A text on organic waste conversion (Bewick 1980), as well as several review articles, discusses the problem of distillery wastewater (Costa Ribeiro and Castello Branco 1980; Sheehan and Greenfield 1980; Weathers 1995; Wilkie et al. 2000). There are many potential solutions for these problems, including simple disposal, sewage treatment, and recovery of useful ingredients. In addition, many attempts have been made to exploit the organic compounds from stillages in biotechnological processes for the production of commercially valuable products. Industrial and experimental utilization and disposal

of effluents and properties of stillage of different origins have been reviewed and compared by Wilkie et al. (2000).

The presence of a large amount of organic substances of natural origin opens up the possibility of bioconversion by microorganisms. While bacteria participate in anaerobic processes producing biogas, fungi are suitable for aerobic bioconversion. There have been several attempts to use fungi for treating distillery wastewaters. The aim of microbiological treatment is to purify the effluent by consumption of organic substances, thus, reducing its COD and BOD, and at the same time to obtain some valuable product, such as fungal biomass for protein-rich animal feed, or some specific fungal metabolite. The present review deals with the application of fungi for treating distillery stillages or slops. Suitable fungi, the bioconversion process, possible products and effects, together with economic considerations are described.

2 DISTILLERY WASTE

Distillery stillages have a complex organic and inorganic composition. The most important characteristics, in terms of their organic strength and nutrient content are given in Table 1.

As can be seen, the values of BOD and COD may vary significantly and may reach extremely high values of over 300 and more than 150 g/l, respectively. The pH ranges from 3 to 5. The waste can contain different amounts of solids up to more than 400 g/l. For cultivating microorganisms, the content of carbon and nitrogen sources is the most important factor. The ratio of C:N varies with the raw material used for alcoholic fermentation. In addition to the components given in Table 1, there are different amounts of organic and amino acids, polyols, vitamins, phenolics, etc. Minerals include potassium, phosphorus, sulfates, and some heavy metals. These components may also have an important influence on

Table 1 Characteristics and composition of distillery stillages

Origin	BOD (g/L)	COD (g/L)	pH	Solids (g/L)	Nitrogen (g/L)	Protein (g/L)	Reducing sugars (g/L)	Total sugars (g/L)
Molasses	16–103	32–158	4.2–5.2	31–119	0.2–6.0	3.7–8	2–18	6–30
Grain	10–340	15	3.2–7.5	20–75	0.2–1.9	5–23	11–30	2.9 ^a –5
Malt	24–43	35–58	2.9–3.8			15–31		6–21 ^a
Cassava	19	23	5.0	24	0.4	2.5	6.8	
Rice	25–84	51	3.5–4.3	94		19.3–20	1.1–13	2.6–9
Barley			3.9			31	20	14
Sweet potato		20	4.3		2.0	4.4	2.4	11
SWL	40–50			100		1		8
Wine	12–18	26	3.6–4.5	23–125	0.4–1.0			0.8–1.1
Fruit		38–156	3.3–4.5	30–440	0.4–1.0			

SWL, sulfite waste liquor.

^a Carbohydrate.

the pollution load of the wastewater as well as on the suitability for bioconversion.

3 FUNGI FOR BIOCONVERSION OF DISTILLERY WASTE

The selection of the proper organisms to be used for stillage bioconversion is of the utmost importance. The organism has to be able to utilize the substrate effectively and at the same time its biomass has to have a high nutritive value, especially in its protein content. According to Tomlinson (1976a) the lower and higher fungi are the most suitable for bioprocessing distillery wastes.

3.1 Yeasts

Yeasts have been chosen in the great majority of cases for cultivation in distillery stillages of different origin. They have been commonly used in food preparation for thousands of years so there are no prejudices regarding their use for food or feed, as is the case with bacteria and molds. The quick growth of yeasts lead to advantages of time and low cost, as well as limited possibility of contamination by other microorganisms. The fermentation process is easy to handle and continuous cultivation is possible. The use of yeast is interesting because of its low price and its high protein and vitamin content. However, some facts must be taken into account (Huyard et al. 1986): (a) the yeast should not contain high levels of contaminants, if it is to be used for feed, (b) energy is needed for separating the yeast from the substrate, (c) effective aeration is necessary, and (d) the remaining effluent still has a high COD and needs to be treated in a sewage treatment facility.

Candida (*Torula*) is the fungal organism most used for molasses stillages and to a lesser extent for other distillery effluents. Other yeasts are also suitable for bioconversion of

molasses stillage. In screening for a useful yeast, *Hansenula*, *Debaromyces*, and *Rhodotorula* were selected out of 203 strains (Akaki et al. 1981). *Hansenula* spp. (Moriya et al. 1990; Shojaosadati et al. 1999) and *Debaromyces* sp. (Selim et al. 1991) were also successful as were *Saccharomyces cerevisiae* (Selim et al. 1991), *Kluyveromyces* spp. (Braun and Meyrath 1981; Selim et al. 1991), *Pichia tainania* (Chang and Yang 1973; Lin et al. 1973) and *Phaffia rhodozyma* (Fontana et al. 1997). *Rhodotorula glutinis* produced biomass for fodder rich in vitamin B on raisin vinasse (Aran 1977; Yazicioğlu et al. 1980). A distillery effluent from rice spirit production could be successfully reused by a *S. cerevisiae* strain (Yang and Tung 1996).

3.2 Filamentous Fungi

Compared to yeasts, filamentous fungi have been used less frequently for bioconversion of distillery wastes. The reasons might be that they are slower growing and more susceptible to infection. However, filamentous fungi have some advantages as they produce a series of extracellular hydrolytic enzymes and are therefore able to exploit complex carbohydrates without prior hydrolysis; this property enables them to grow in starch and cellulose substrates. The second advantage is in their filamentous morphology that allows separation of the biomass by simple filtration. Compared to yeasts, filamentous fungi have lower sensitivity to variations in temperature, pH, nutrients, and aeration, and they also have a lower nucleic acid content in the biomass (Araújo et al. 1977). Various species of fungi were used for distillery stillage fermentations, among them *Aspergillus* spp. being the most popular ones. *Aspergillus oryzae* (Araújo et al. 1977; De Lamo and De Menezes 1978) and *Aspergillus niger* (De Lamo and De Menezes 1978) were selected out of several filamentous fungi due to their high biomass yield, rapid filtration, and substantial COD reduction in cane molasses stillage. *A. niger* was also suitable for bioconversion of rice spirit

(Yang and Lin 1998). *Aspergillus awamori* var. *kawachi* and *Aspergillus usami* mut. *shirousami* were used for bioconversion of distillery waste of a Japanese alcohol beverage, *shochu* (Kida et al. 1995; Morimura et al. 1991; 1992; 1994a). Filamentous fungi were chosen for conversion of stillage, due to their texture, protein profile, and easy harvesting, and *Aspergillus phoenicis* was the most promising among isolates from contaminated stillage (De Gonz  les and De Murphy 1979). *Aspergillus* species were also suitable for converting fruit based distillery stillages, *A. niger* biomass produced in vinasse from raisin brandy has a good amino acid content (Aran 1977; Yazicio  lu et al. 1980), while cellulolytic *Aspergillus* species were very effective in improving filtration and reducing COD of apple brandy slops (Friedrich et al. 1983; 1986; 1987; Gunde-Cimerman et al. 1986; Perdih et al. 1991). Many other fungi proved promising in treating and converting different kinds of stillages. For example, *Penicillium* species were chosen for utilization of white wine vinasse, due to their good adaptation, utilization of the medium, and development at low pH values (Magny et al. 1977). *Penicillium oxalicum* produced the highest biomass amount with high protein content in raisin brandy vinasse (Aran 1977; Yazicio  lu et al. 1980). *Geotrichum candidum* was selected from a variety of soil microorganisms for whisky stillage conversion (Quinn and Marchant 1979a,b; 1980) and for decolorizing molasses stillage, since it was able to reduce the phenolic compounds (Fitzgibbon et al. 1995; 1998). Compared to several yeasts, molds, and bacteria, *Paecilomyces varioti* was very effective in aerobic treatment (Nudel et al. 1987) and for producing single cell protein (SCP) from cane molasses stillage (Azzam and Heikel 1989; Cabib et al. 1983). *Gliocladium deliquescens* performed best among different fungi in rum distillery wastewater (Rolz et al. 1975). *Acremonium fusidioides* was isolated from soil, treated with stillage, and it clarified and decolorized the effluent and produced the highest biomass amount (Rosalem et al. 1985). *Trichoderma* species were successfully utilized in aerobic treatment of cane molasses stillage (Nudel et al. 1987) and for bioconversion of fruit distillery slops; the fungi degraded raw fibers, exhibited high cellulolytic activity, and produced high protein content in the biomass (Friedrich et al. 1986; Perdih et al. 1991). *Fusarium moniliforme* grown in vinasse from raisin brandy produced high lipid biomass (Aran 1977; Yazicio  lu et al. 1980). *Myrothecium verrucaria* was effective in reducing organic substances in apple distillery slop as well as in improving its filterability (Friedrich et al. 1986). *Aureobasidium* sp. could produce a biopolymer pullulan from corn condensed distiller's solubles (Leathers and Gupta 1994). Among the microorganisms tested for the treatment and bioconversion of *shochu*, a Japanese distilled spirit, zygomycetous fungi, *Absidia atrospora* and *Gongronella butleri* strains were selected and used in a cost effective procedure for chitosan production in the wastewater (Yokoi et al. 1998).

The development of basidiomycetous fungi (mushrooms) in submerged culture is interesting because of their probable suitability as cheap substitutes for mushroom fruiting bodies

in certain kinds of food. Falanghe (1962) investigated the suitability of stillage for growing mushroom mycelia as a source of protein and fat. Among ten strains cultivated in submerged conditions, *Agaricus campestris* and *Boletus indecisis* were chosen as the two most suitable. *A. campestris* was more effective in producing mycelial protein content. *B. indecisis*, however, exhibited a greater ability for mycelial production. The dried mycelia had a pleasant slight flavor and they seemed to have adequate characteristics for improving foods; their growth in pellet form enabled easy separation from the medium. In apple distillery slop, *Phanerochaete chrysosporium* grew successfully; it reduced the amount of fiber and improved protein content in the biomass (Friedrich et al. 1986). *Coriolus versicolor* and *P. chrysosporium* could be used for reducing pollution and decolorizing the diluted molasses spent wash (Fitzgibbon et al. 1995; 1998). Other white rot fungi, *Funalia trogii* and *Trametes versicolor* were used for producing plant growth hormones in vinasse from molasses and, in addition, partial COD and color were removed (Y  rekli et al. 1999).

3.3 Mixed Cultures

Some research was done on the use of mixed cultures of different microorganisms to improve the utilization of nutrients in stillage and to obtain better yields of biomass. Combinations of different yeasts, yeasts and bacteria, yeasts and molds or different molds were studied. Biomass yield of the mixed cultures can be substantially higher than that of pure cultures. A two-step fermentation proved to be very effective. Yeast was used in the first step to produce large amounts of biomass, and a filamentous fungus in the second step markedly reduced the COD of the effluent (Azzam and Heikel 1989; Bottaro Castilla et al. 1984; Nudel et al. 1987). In malt whisky spent wash a three-membered mixed culture of *G. candidum*, *Hansenula anomala*, and *Candida krusei* constituted a stable population. The relative proportions of the three organisms were dependent on the dilution rate during continuous cultivation (Barker et al. 1982). Thirteen different yeast species, mainly *Candida* spp. and *Trichosporon* spp., were used in a mixed culture for treating the vinasse of beet molasses (Malnou et al. 1987). Mixed cultures of two ascomycetous fungi, *A. awamori* and *Trichoderma reesei*, were tested in apple slops; they expressed the positive properties of both genera: *Aspergillus* improved the filtration time and COD reduction whereas *Trichoderma* decreased fiber and increased protein content in the biomass (Friedrich et al. 1987).

4 BIOCONVERSION PROCESS

The stillage may be used as a fermentation medium directly after distillation, or it may be pretreated by clarification, dilution, pH adjustment, and sterilization. In certain cases, supplementation with additional sources of nitrogen and/or

Table 2 Products and effects of bioconversion of distillery waste by yeasts

Cultivation	Yeast species	Stillage	Biomass (g/L)	Protein (%)	COD reduction (%)	References
Batch	<i>C. utilis</i>	CMS	9–18	28–50	46	Several references
	<i>C. utilis</i>	CMS suppl.	4–25	30–52	16–52	Several references
	<i>C. arborea</i>	CMS	7–13	33–43	12–22	Matsuo et al. (1965)
	<i>C. brumptii</i>	CMS suppl.	10	53	20	Kim et al. (1976)
	<i>C. krusei</i>	CMS suppl.	3–8	31–48		Tauk (1982) and Tauk and Gambale (1981)
	<i>C. membranefaciens</i>	CMS suppl.	3.6	45		Tauk and Gambale (1981)
	<i>C. rugosa</i>	BMS suppl.	14.7		45.5	Lee and Baerwald (1991)
	<i>C. steatolytica</i>	HHS	12			Zhang et al. (1982)
	<i>C. stellatoidea</i>	CMS suppl.	3.5	48		Tauk and Gambale (1981)
	<i>C. tropicalis</i>	WV		48		Karova et al. (1976)
Fed batch	<i>P. tainania</i>	CMS suppl.	19–20	42–49		Lin et al. (1973)
	<i>S. cerevisiae</i>	MS suppl.	12.7			Selim et al. (1991)
	<i>S. cerevisiae</i>	Rice	6			Yang and Tung (1996)
	<i>S. cerevisiae</i>	Rice suppl.	20			Yang and Tung (1996)
	<i>C. utilis</i>	CMS suppl.	15	41		Cabib et al. (1983)
Continuous	<i>C. utilis</i>	CMS suppl.	4–24	40–52	30–40	Several references
	<i>Hansenula</i> sp.	BMS	5.7	39.6	31	Shojaosadati et al. (1999)
	<i>Hansenula</i> sp.	BMS suppl.	8.5	50.6	35.7	Shojaosadati et al. (1999)
	<i>K. marxianus</i>	BMS	10–11		60–70	Braun and Meyrath (1981)
	<i>S. cerevisiae</i>	Rice suppl.	70			Yang and Tung (1996)

CMS, cane molasses stillage; suppl., supplemented; BMS, beet molasses stillage; WV, wine vinasse; HHS, hemicellulose hydrolyzate stillage; MS, molasses stillage of unknown origin.

phosphorus may be necessary. Sometimes enrichment of stillage with an easily metabolizable carbon source can improve growth and substrate utilization (Cabib et al. 1983; Wang et al. 1977). The proportions of elements C:N:P of 50:5:1 were used in growing filamentous fungi in sugarcane stillage (Araújo et al. 1977; De Lamo and De Menezes 1978), while 40:10:1 was optimal for *A. fusidioides* in the same substrate (Rosalem et al. 1985). Cells from agar slants can be used directly as inocula for stillage fermentation, or the relevant microbes can be precultured under submerged conditions. The usual quantity of inoculum for inoculation is 10% of the broth volume. Previous adaptation of the microbial strain to the stillage may have a positive effect on bioconversion. Submerged aerobic processes are used with laboratory experiments carried out in shake flasks and fermentors. In large-scale experiments culture volumes of 9 m³ (Cabib et al. 1983) and even 80 m³ (Wang et al. 1980) have been reported. Batch, fed batch, or continuous operation modes are employed in trial fermentation. In addition to single fungus inocula, mixed cultures of microorganisms or a two-stage cultivation with successive cultivation of two different microorganisms have been used. Cell recycling proved to be beneficial for higher yields of biomass as well as for better consumption of organic substances (Staheeu et al. 1985; Wang et al. 1980). Cultivation temperatures were kept between 22 and 38°C, suitable for mesophilic microorganisms; pH control at the predetermined value often proved to be beneficial to the microorganism. The fermentation broth required vigorous aeration to ensure good growth. Aeration values of 0.5–1.0 vvm generally were employed; exceptions being the very high aeration rates in small bioreactors or lower aeration rates of 1 vvh (Wang et al. 1980) in large scale experiments. Agitation rates range from 200 to 1000 rpm. In continuous cultivation, dilution rates of 0.016 h⁻¹ (Yang and Tung 1996) to 0.383 h⁻¹ (Wang et al. 1980) were employed. The dilution rate could be increased by the use of a cell-recycling technique (Wang et al. 1980). In batch processes the fermentation time is dependent on the microorganism and ranged from several hours to 2 days with yeasts, to several days with molds, and up to 12 days with mushrooms. After fermentation the yeast biomass is generally harvested by centrifugation, whereas with filamentous fungi simple filtration is possible.

5 PRODUCTS AND EFFECTS

Bioconversion of distillery waste by means of fungi brings a double benefit: the effluent is substantially purified and, in addition, it is possible to obtain useful products, such as protein-rich fungal biomass, ethanol, enzymes, etc. Results of bioconversion by yeasts are given in Table 2.

In most experiments *C. utilis* was used as the chosen microorganism, especially for cane molasses stillage (Friedrich et al. 1992). The quantity of cells produced varied significantly with the concentration of the substrate and with the addition of N sources. With increasing nitrogen

supplementation a concentration of biomass of up to 25 g/l could be obtained with *C. utilis* in cane molasses stillage (Cabib et al. 1983). The high quantity of *C. utilis* biomass of 22 g/l obtained by Wang et al. (1980) in continuous fermentation was a result of cell recycling. Crude protein content in the biomass of *Candida utilis* accounted for 28–58% of the cell mass. With the same species the maximal COD reduction in the effluent was 52% (Nudel et al. 1987) and the highest BOD reduction was 55% (Matsuo et al. 1965).

Among yeasts shown in Table 2, other than *C. utilis*, outstanding biomass concentration of 70 g/l was produced by *S. cerevisiae* during continuous cultivation in enriched rice spirit stillage (Yang and Tung 1996). In general, concentrations of about 20 g/l were relatively high. The biomass of *Candida brumptii* showed the highest protein content of 53%. *Kluyveromyces marxianus* seemed to be outstandingly efficient, considering the COD reduction of 60–70%, while its biomass concentration of 10 g/l was low in comparison with *C. utilis*. The results of reduced COD and BOD are given in terms of relative values and the absolute reduction is dependent on the initial value.

Filamentous fungi are cultivated mostly in batch processes. Results of bioconversion of distillery waste by filamentous fungi are given in Table 3.

When compared with yeasts, filamentous fungi are more effective in consuming polluting substances, since COD and BOD reductions of over 80 and 90%, respectively, have been recorded. *A. oryzae* (Araújo et al. 1977) and *G. deliquescens* (Rolz et al. 1975) in sugarcane stillage, *Penicillium* strains in wine vinasse (Magny et al. 1977), and *G. candidum* in malt whisky stillage (Quinn and Marchant 1980) proved to be the most promising in this respect. The highest amount of biomass was reached with *A. awamori* var. *kawachi* in rice-shochu distillery wastewater (Morimura et al. 1994a). It was observed that on average the protein content in the biomass of filamentous fungi was lower than that of yeasts. *G. candidum*, however, was found to produce the highest protein content; there was 45.5% “true” protein in the biomass as determined by the biuret method (Quinn and Marchant 1980).

An overview of the bioconversion products and effects using mixed cultures of different microorganisms is given in Table 4.

In general, mixed cultures were the most effective in substrate utilization, based on COD values. A co-culture of 15 yeast strains in a high-loaded beet molasses stillage of initial 74.5 g/l COD, produced the highest amount of biomass, 28.9 g/l, in batch culture. When diluted medium was applied, the biomass yield was much lower (Huyard et al. 1986). A successful approach seems to be a two-step separated cultivation of *C. utilis* and *P. varioti* by which a total amount of 22 g/l cell material and a COD reduction of as much as 90% were obtained. During the first step, *C. utilis* was used mainly for SCP production, whereas *P. varioti* in the second step consumed the reducing organic substances in the liquid phase (Azzam and Heikel 1989; Bottaro-Castilla et al. 1984). Similar results were observed in a two-step continuous cultivation of *C. utilis* and *A. niger* grown separately (Nudel

Table 3 Products and effects of bioconversion of distillery waste by filamentous fungi

Fungus	Stillage	Biomass (g/L)	Protein (%)	COD reduction (%)	BOD reduction (%)	References
<i>A. campestris</i>	CMS	13	45			Falanghe (1962)
<i>A. fusidioides</i>	CMS	11	40			Rosalem et al. (1985)
<i>A. niger</i>	CMS	8–13	30–40	46–78		De Lamo and De Menezes (1978) and Rosalem et al. (1985)
<i>A. awamori</i> var. <i>kawachi</i>	Rice	40	40		76 (TOC)	Morimura et al. (1994b)
<i>A. niger</i>	Fruit	4–20	12–36	50–70		Friedrich et al. (1983, 1986) and Gunde-Cimerman et al. (1986)
<i>A. oryzae</i>	CMS	14–17	35–50	61–88	79–83	Araújo et al. (1977)
<i>A. oryzae</i>	CMS	12–15	39	48–72	78	De Lamo and De Menezes (1978)
<i>A. phoenicis</i>	CMS (rum)	20	21		58	De Gonzáles and De Murphy (1979)
<i>G. candidum</i>	Whisky ^a	3.5		80.6	92	Quinn and Marchant (1980)
<i>G. candidum</i>	Whisky	11–27		32–78	63–91	Quinn and Marchant (1980)
<i>G. candidum</i>	Whisky ^b	34	46		87	Quinn and Marchant (1980)
<i>G. deliquescens</i>	CMS (rum)	11–19		60–85		Rolz et al. (1975)
<i>M. verrucaria</i>	CMS (rum)	11–12		79–82		Rolz et al. (1975)
<i>P. elegans</i>	CMS (rum)	11–14		62–66		Rolz et al. (1975)
<i>P. varioti</i>	CMS	13–25	40	43–70		Bottaro Castilla et al. (1984), Cabib et al. (1983), and Nudel et al. (1987)
<i>P. varioti</i>	CMS	5		70		Azzam and Heikel (1989)
<i>P. oxalicum</i>	Raisins	12	34			Aran (1977) and Yazicioğlu et al. (1980)
<i>Penicillium</i> sp.	CMS (rum)	12–16		65		Rolz et al. (1975)
<i>Penicillium</i> spp.	WV	13	41		91	Magny et al. (1977)
<i>T. viride</i>	CMS	12–28		59–79		Nudel et al. (1987) and Rolz et al. (1975)

CMS, cane molasses stillage; WV, wine vinasse.

^a Diluted five times.^b Continuous, two stage.

Table 4 Bioconversion of distillery waste by mixed microbial cultures

Microorganisms	Stillage	Biomass (g/L)	COD reduction (%)	Cultivation mode	References
<i>P. varioti</i> + <i>T. viride</i>	CMS	12–17	50–64	Batch	Nudel et al. (1987)
<i>T. viride</i> + <i>A. oryzae</i>	CMS	10–13	50–54	Batch	Nudel et al. (1987)
<i>C. utilis</i> + <i>P. varioti</i>	CMS	8–12	45–50	Batch	Nudel et al. (1987)
<i>C. utilis</i> + <i>C. acetobacterophilum</i>	CMS	16–17	65	Batch	Nudel et al. (1987)
<i>C. utilis</i> + <i>B. flavum</i>	CMS	16	65	Batch	Nudel et al. (1987)
<i>Azotobacter</i> + <i>C. utilis</i>	CMS	16		Batch	Nudel et al. (1987)
<i>C. utilis</i> + <i>A. niger</i>	CMS	16–17	89	Cont. serial	Nudel et al. (1987)
<i>C. utilis</i> + <i>P. varioti</i>	CMS	22	92	Batch	Bottaro Castilla et al. (1984)
<i>C. utilis</i> + <i>P. varioti</i>	MS	22	90	Batch, two step	Azzam and Heikel (1989)
<i>C. utilis</i> + <i>P. varioti</i>	CMS	14–16	85	Cont. serial	Bottaro Castilla et al. (1984)
15 yeasts	BMS dil.	7	79	Batch	Huyard et al. (1986)
15 yeasts	BMS dil.	9	72	Cont.	Huyard et al. (1986)
15 yeasts	BMS	29	74	Batch	Huyard et al. (1986)
13 yeasts	BMS dil.	7–12	68–75	Cont.	Malnou et al. (1987)
<i>G. candidum</i> + <i>C. crusei</i> + <i>H. anomala</i>	MWS	13	55	Batch	Barker et al. (1982)
<i>G. candidum</i> + <i>C. crusei</i> + <i>H. anomala</i>	MWS	5	32	Cont.	Barker et al. (1982)
<i>A. awamori</i> + <i>T. reesei</i>	Apple dil.	5	31	Batch	Friedrich et al. (1987)

CMS, cane molasses stillage; BMS, beet molasses stillage; MS, molasses stillage (unknown origin); dil, diluted; MWS, malt whisky stillage.

Table 5 Bioconversion of stillage by continuous cultivation of fungi

Fungus	Dilution rate (h ⁻¹)	Productivity (g/L/h)	Biomass (g/L)	COD reduction (%)	BOD reduction (%)	References
<i>C. utilis</i>	0.383	4.24	17.9		34.6	Wang et al. (1980)
<i>C. utilis</i>	0.365	4.06	21.9		37.1	Wang et al. (1980)
<i>C. utilis</i>	0.22	2.64	12			Cabib et al. (1983)
<i>C. utilis</i>	0.27	3.24	12			Cabib et al. (1983)
<i>C. utilis</i>	0.2	1.8–2.0	9–10	35		Bottaro Castilla et al. (1984) and Nudel et al. (1987)
<i>C. utilis</i>	0.2					
<i>P. variot</i> ^a	0.1	2.3–2.6	19–21	85		Bottaro Castilla et al. (1984)
<i>C. utilis</i>	0.2					
<i>A. niger</i> ^a	0.1	2.7–3.2	15–17	89		Nudel et al. (1987)
<i>K. marxianus</i>	0.3	3	10–11	60–70		Braun and Meyrath (1981)
<i>G. candidum</i>	0.125	2.24		30	50	Quinn and Marchant (1980)
<i>G. candidum</i> 1st step	0.125					
2nd step	0.10		31		50	Quinn and Marchant (1980)
<i>G. candidum</i> 1st step	0.125					
2nd step	0.085	1.72	34		87	Quinn and Marchant (1980)
<i>G. candidum</i> + <i>C. crusei</i> + <i>H. anomala</i>	0.10	0.48	4.8	31.5		Barker et al. (1982)
<i>G. candidum</i> + <i>C. crusei</i> + <i>H. anomala</i>	0.20	0.36		15.9		Barker et al. (1982)
<i>G. candidum</i> + <i>C. crusei</i> + <i>H. anomala</i>	0.35	0.42		2.0		Barker et al. (1982)
<i>Hansenula</i> sp.	0.12	~1	8.5	35.7		Shojaosadati et al. (1999)
<i>G. deliquescens</i>	0.03		17–22	61–50		Rolz et al. (1975)
<i>S. cerevisiae</i>	0.016		70 ^b			Yang and Tung (1996)
Association of 15 yeasts	0.133–0.134	0.5–1.6	11	68		Huyard et al. (1986) and Malnou et al. (1987)

^a Two step process.^b Enriched stillage.

et al. 1987). Summarizing the results of conversion of both steps, 17 g/l biomass and 89% COD reduction were obtained. In general, it was observed that the combination of yeast with filamentous fungi resulted in improvement of COD reduction, and improved wastewater purification.

While the batch cultivation mode is most frequently used, continuous cultivation of fungi in stillages can have some advantages regarding the product yield (Table 5).

As shown in Table 5, an outstanding biomass yield of 70 g/l was produced by *S. cerevisiae* in enriched stillage from rice spirit distillation when the dilution rate was low (Yang and Tung 1996). On the other hand, dilution rates of over 0.36 h^{-1} with cell recycling resulted in a productivity of over 4 g/l/h *Candida* biomass and a steady-state biomass concentration of 18–22 g/l (Wang et al. 1980). In continuous culture, COD was reduced most effectively by using *K. marxianus* (Braun and Meyrath 1981). A serial culture of a yeast and a filamentous fungus in two steps gave the most promising results with respect to both biomass yield and efficient consumption of organic matter (Bottaro Castilla et al. 1984; Nudel et al. 1987). Two-step cultivation of *G. candidum* also seemed to be very suitable, with the steady-state biomass of 31–34 g/l (Quinn and Marchant 1980). The continuous cultivation process could lead to a higher protein content in the biomass and enhance the stability of the culture as well as its resistance to contamination (Barker et al. 1982).

Microbial biomass rich in protein is the main product of fungal bioconversion of stillages. Some information is available about cell material composition. It appears that *C. utilis* biomass composition is similar to that of other yeasts grown in carbohydrate media. Microbial protein has a good balance of amino acids with the exception of those containing sulfur, such as cysteine and methionine; the content of the latter amino acids is generally low in microbial biomass (Cabib et al. 1983; Quinn and Marchant 1979b; Yazicioğlu et al. 1980). However, lysine is in excess when compared to the recommended level of the FAO standards (Quinn and Marchant 1979b). Yeast biomass from vinasse is rich in nitrogen, vitamins, and other biologically active substances. The vitamin B complex of yeast is very efficient and could not be adequately substituted by a mixture of analytically pure vitamins (Cabib et al. 1983; Yazicioğlu et al. 1980).

Nutritional evaluation of yeast biomass grown in malt whisky distillery slop showed that it was suitable for nonruminants with a net protein utilization value of 0.40, and a digestibility of 0.67. The biomass was not toxic, as confirmed by toxicological tests (Barker et al. 1982). When molasses stillages were used, K and Mg contents could have laxative effects (Araújo et al. 1977). The nucleic acid content in cell material should be as low as possible; filamentous fungi have an advantage over yeasts in this respect (Araújo et al. 1977). It has been observed that levels of DNA and RNA were lower in batch than in continuous cultures (Quinn and Marchant 1979b). Feeding experiments of *Aspergillus* biomass in a diet for chicks, demonstrated excellent

acceptability, good weight gain, and no toxicity; the biomass had a very good protein efficiency ratio, being comparable to meat and soya meal (Araújo et al. 1977). A 1% methionine addition to *Candida* yeast biomass increased the biological value, which was not much lower than that of casein (Cabib et al. 1983).

With the use of fungi for bioconversion of distillery wastewaters, not only fungal biomass but also other products can be obtained. Secondary ethanol can be produced by the original yeast used in a primary alcohol fermentation, if the distillation is performed at low temperatures in vacuum (Teramoto et al. 1993; Ueda and Teramoto 1995; Ueda et al. 1991). Microbial polysaccharides can be produced, such as pullulan with *Aureobasidium* sp. (Leathers and Gupta 1994) or chitosan with *A. atrospora* or *G. butleri* strains (Yokoi et al. 1998). Hydrolytic enzymes can be produced by filamentous fungi. Cellulases and other glucosidases can be products of bioconversion when cellulolytic fungi are grown in fruit distillery slops (Friedrich et al. 1986; 1987; Gunde-Cimerman et al. 1986). Some ascomycetes, especially *A. awamori* var. *kawachi*, are able to produce starch saccharifying enzymes from *shochu* distillery wastewater (Morimura et al. 1991; 1992). Acid proteases are produced mainly with *Aspergillus* species in rice (Morimura et al. 1994a; Yang and Lin 1998) and barley (Morimura et al. 1994a) *shochu* stillages. Possible products are a yeast pigment, astaxanthin, from *P. rhodozyma* (Fontana et al. 1997) and plant growth hormones, obtained by growing white rot fungi, such as *F. troglia* or *T. versicolor* (Yürekli et al. 1999).

6 ECONOMIC CONSIDERATIONS

Little information is available concerning the economics of the bioconversion process. Only rough estimates of costs were given by Maiorella et al. (1983) and Tomlinson (1976b). The main costs are high capital investment (Tomlinson 1976b), energy needed for aeration, agitation and cooling (Maiorella et al. 1983), biomass separation (Tomlinson 1976b), nutrient supplementation, sterility problems, limited production, and irregularities in raw material composition. However, capital cost may not be too high, as was shown in the cost evaluation for molasses stillage treatment methods, where aerobic yeast growth was estimated to require the lowest capital investment (Maiorella et al. 1983). Separation of cell materials from the broth would be easy with the application of filamentous fungi. The addition of nutrients is not always required. Sterility problems could be managed or at least reduced by maintaining a low pH (Cabib et al. 1983; Huyard et al. 1986; Matsuo et al. 1966), and the use of mixed cultures (Barker et al. 1982). The problem with large-scale plants is the need for a continuous and relatively homogeneous supply of stillage. Comparison of cost with that for conventional biological treatment has shown that annual savings could be increased by applying fungal bioconversion of stillages (Tomlinson 1976b). Similarly, rough estimates of capital and operating costs for molasses stillage treatment by fungi show

that savings could be gained, even though wastewater would still have to be purified. Comparison of different treatment methods for molasses stillage showed that aerobic *Candida* yeast growth was the most suitable option. The same method was proposed for sulfite waste liquor and wood hydrolysis stillages (Maiorella et al. 1983).

7 CONCLUSIONS

The use of fungi for bioconversion of distillery waste into microbial biomass or some useful metabolites is reviewed. Stillages remaining in alcohol distilleries, although based on different raw materials have the common characteristic in that they are highly loaded effluents, which are difficult to purify. They are harmful to the environment due to the high content of organic substances and low pH. However, these properties facilitate their use as substrates for fungal growth. Sugarcane molasses stillages have been used for the most part. Among fungi, yeasts, especially *C. utilis*, predominate. To a minor extent filamentous fungi, including Basidiomycetes, as well as mixed fungal or mixed fungal and bacterial cultures have been used. Laboratory, pilot scale, and industrial scale experiments have been performed, and batch or continuous, single- or two-step fermentations have been introduced. The end products of bioconversion are fungal biomass and substantially purified and decolorized effluents. A well-balanced amino acid composition makes the biomass suitable as an animal feed supplement. In addition to biomass other products can be obtained, such as ethanol, enzymes, polysaccharides, pigments, and plant growth hormones. The economics of stillage bioconversion with fungi is dependent on many different factors and can vary from country to country. However, the conclusion is that fungi have a great potential in helping to solve pollution problems and, at the same time, producing marketable products.

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Degradation of Hydrocarbons by Yeasts and Filamentous Fungi

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1 INTRODUCTION

Hydrocarbons are ubiquitous in the environment, but they are most abundant where coal and petroleum fuels are stored, processed, or burned (Tuháčková et al. 2001; Warshawsky 1999). Because many hydrocarbons are toxic, their biological effects have been studied extensively. Occupational exposure to polycyclic aromatic hydrocarbons (PAHs) in the aluminum, coke, and steel industries has been linked to lung and bladder cancer (Mastrangelo et al. 1996) and exposure to hydrocarbon fuels may produce neurotoxic effects (Ritchie et al. 2001). Mammalian metabolites of benzo[*a*]pyrene, the most important carcinogen in natural PAH mixtures, form adducts with macromolecules and interfere with cellular signaling pathways (Miller and Ramos 2001). Some PAHs are either weakly estrogenic or antiestrogenic (Santodonato 1997). The widespread ability of yeasts and filamentous fungi to transform hydrocarbons suggests that they may be involved in the recycling of naturally occurring hydrocarbons in the environment as well as in the biodeterioration of liquid fuels (Lindley 1992). Their versatility in degrading hydrocarbons is due to the broad substrate specificity of their enzymes (Cerniglia and Sutherland 2001). Selected fungi are now being exploited for the bioremediation of soils contaminated with hydrocarbon wastes (Atlas and Cerniglia 1995; Cerniglia and Sutherland 2001) and other species are used for the biotransformation of hydrocarbons to higher-value compounds (Trudgill 1994).

The recycling of hydrocarbons by fungi is probably common, though usually unnoticed, in the environment. For instance, certain yeasts can utilize the methane produced in lakes (Wolf and Hanson 1979; 1980). Fungi that are pathogenic to insects can degrade the hydrocarbons found in the epicuticular waxes of their hosts (Crespo et al. 2000;

Napolitano and Juárez 1997). Many filamentous fungi and yeasts grow abundantly in soils contaminated by petroleum residues (April et al. 1998; 2000; Ekundayo and Obuekwe 2000). Fungi also degrade hydrocarbons in streams and lakes (Griffin and Cooney 1979; Romero et al. 2001) and in oil-polluted seawater (Cerniglia 1997; Zinjarde et al. 1998). Filamentous fungi, especially *Hormoconis resinae* [the anamorph of *Amorphotheca resinae*], may contaminate aviation fuels when water is present (Parbery 1971). Yeasts are responsible for biodeterioration of liquid fuels at the interface with water and mycelial fungi may physically block the fuel lines of ships and aircraft (Lindley 1992). The ability of fungi to metabolize hydrocarbons is now being exploited to clean up the environment. Several fungi are used to remove PAHs from wastewater (Giraud et al. 2001; Liao et al. 1997) and yeasts are used to decompose petroleum residues in estuaries (Nwachukwu 2000). Fungi have been used to inoculate contaminated soils to degrade hydrocarbons (May et al. 1997; Novotny et al. 1999; Rama et al. 2000). Cultures that grow on toluene, ethylbenzene, and styrene can be used in biofilters to remove these compounds from industrial waste gases (Cox et al. 1996; García-Peña et al. 2001; Prenafeta-Boldú et al. 2001).

Many purified hydrocarbons can be transformed stereospecifically by fungi to produce higher-value products. The monoterpenes α - and β -pinene (Prema and Bhattacharyya 1962), β -myrcene (Yamazaki et al. 1988), and limonene (Noma et al. 1992; de Oliveira and Strapasson 2000) as well as several sesquiterpenes (Abraham et al. 1992; Miyazawa et al. 1995; 1997; 1998) are transformed to chiral metabolites. (+)-Limonene can be transformed to perillyl alcohol, an anticancer drug (de Oliveira and Strapasson 2000). Ethylbenzene and propylbenzene are transformed stereospecifically to (+)-1-phenylethanol and (+)-1-phenylpropanol,

which may be used as chiral building blocks for chemical synthesis (Uzura et al. 2001). Some species of fungi are more abundant in sites with hydrocarbon deposition (Tuháčková et al. 2001). The fungal genera that have been studied most extensively for use in the biotransformation of hydrocarbons are the white-rot basidiomycetes *Phanerochaete*, *Pleurotus*, and *Trametes*, the zygomycete *Cunninghamella*, the hyphomycetes *Aspergillus* and *Penicillium*, and the yeast *Candida*. Several of these fungi have been used in the experimental bioremediation of soils contaminated with oil (Atlas and Cerniglia 1995; Colombo et al. 1996). *Phanerochaete chrysosporium* causes a white rot of wood by degrading lignin (Hammel 1995). It oxidizes PAHs to *trans*-dihydrodiols, phenols, quinones, glucoside conjugates, aromatic acids, and eventually CO₂ (Bumpus 1989; Cerniglia and Sutherland 2001; Sutherland et al. 1991). Several enzymes are responsible, including lignin peroxidase, manganese peroxidase, and cytochrome P450 (Bogan et al. 1996; Cerniglia and Sutherland 2001; Hammel et al. 1991; 1992). *P. chrysosporium* also cometabolizes single-ring aromatic compounds, such as benzene and toluene, to CO₂ (Yadav and Reddy 1993). *P. laevis*, another species which oxidizes PAHs to polar metabolites (Bogan and Lamar 1996), produces manganese peroxidase and a small amount of laccase but not lignin peroxidase.

Pleurotus ostreatus, the cultivated oyster mushroom, is another white-rot fungus with the ability to mineralize PAHs to CO₂ (Bezalet et al. 1996a; Wolter et al. 1997). Other metabolites include *trans*-dihydrodiols, quinones, and aromatic acids (Bezalet et al. 1996b). Evidence supports the involvement of a cytochrome P450 and an epoxide hydrolase in the early steps of PAH metabolism; manganese peroxidase and laccase may be involved in the later steps (Bezalet et al. 1996b; Schützendübel et al. 1999). *P. pulmonarius*, a related species, produces a cytochrome P450 that hydroxylates benzo[*a*]pyrene (Masaphy et al. 1999). *Trametes versicolor*, another basidiomycete that causes a white rot of wood, also degrades PAHs in soil (Rama et al. 2000). It produces a manganese peroxidase that oxidizes fluorene and phenanthrene (Collins and Dobson 1996). *T. versicolor* also produces laccases, which can oxidize anthracene and benzo[*a*]pyrene in the presence of chemical mediators (Collins et al. 1996; Johannes and Majcherzyk 2000). A second species, *T. hirsuta*, produces a laccase that has been shown to oxidize phenanthrene in the presence of linoleic acid and the mediator 1-hydroxybenzotriazole (Böhmer et al. 1998). *Cunninghamella elegans*, a zygomycete, oxidizes biphenyl and a large variety of PAHs to metabolites with higher water solubility (Cerniglia 1992; 1993; 1997; Dodge et al. 1979; Cerniglia et al. 1992; Sutherland 1992; Pothuluri and Cerniglia 1994; Cerniglia and Sutherland 2001). *C. elegans* first oxidizes a PAH to an arene oxide by a cytochrome P450 monooxygenase reaction (Cerniglia 1992; 1997). It then quickly hydrates the arene oxide, forming a *trans*-dihydrodiol, by an epoxide hydrolase reaction (Sutherland et al. 1995). Alternatively, the arene oxide may rearrange nonenzymatically to form a phenol. The *trans*-dihydrodiols and phenols may be

conjugated later with sulfate or glucoside (Casillas et al. 1996; Pothuluri et al. 1990; 1996). Another species, *C. blakesleeana*, oxidizes cyclohexylcyclohexane (Davies et al. 1986).

Aspergillus niger and *A. fumigatus* both metabolize terpenes and PAHs. *A. niger* converts the terpene β -myrcene to dihydroxylated derivatives (Yamazaki et al. 1988). Another strain of this species oxidizes the PAHs phenanthrene and pyrene to phenols and methyl ethers (Sack et al. 1997a), and there is even a report of the ability of *A. niger* to cleave the rings of naphthalene, anthracene, and phenanthrene (Yogambal and Karegoudar 1997). *A. cellulosa* [*A. fumigatus*] transforms (+)- and (-)-limonene by hydroxylation, double-bond reduction, and ketone formation (Noma et al. 1992). *A. fumigatus* also produces a cytochrome P450 that hydroxylates benzo[*a*]pyrene (Venkateswarlu et al. 1996). *Penicillium glabrum* oxidizes pyrene to mono- and dihydroxylated derivatives, quinones, methyl ethers, and a sulfate (Wunder et al. 1997). *P. digitatum* hydrates one of the double bonds in (+)-limonene to form (+)- α -terpineol (Demyttenaere et al. 2001; Tan et al. 1998) and a different *Penicillium* sp. strain transforms α -pinene to verbenone (Agrawal and Joseph 2000). *P. janthinellum* oxidizes high-molecular-weight PAHs to phenols and quinones (Boonchan et al. 2000; Launen et al. 2000). *Candida maltosa*, a yeast that grows on *n*-alkanes, and some other *Candida* spp. produce cytochrome P450 monooxygenases that hydroxylate *n*-hexadecane and *n*-octadecane (Scheller et al. 1996). Alkane-utilizing *Candida* sp. strains from oil-polluted soils may produce biosurfactants as well as oxidative enzymes (Ekundayo and Obuekwe 2000). A strain of *C. utilis* that utilizes *n*-alkanes has been used for the bioremediation of mangrove sites (Nwachukwu 2000). Among the hydrocarbons that can be transformed by fungi are alkanes (Lindley 1992; Morgan and Watkinson 1994), terpenes (Trudgill 1994), monocyclic aromatic compounds (Prenafeta-Boldú et al. 2001; Yadav and Reddy 1993), and PAHs (Cerniglia 1992; 1993; 1997; Cerniglia and Sutherland 2001; Cerniglia et al. 1992; Juhasz and Naidu 2000; Hammel 1995; Münchnerová and Augustin 1994; Pothuluri and Cerniglia 1994; Sutherland 1992; Sutherland et al. 1995). This chapter will emphasize the recent research on hydrocarbon transformation by fungi.

2 ALKANES AND ALKENES

Many soil fungi, such as *Aspergillus terreus*, *Pseudallescheria boydii*, and the yeast *Candida tropicalis*, degrade the mixtures of *n*-alkanes found in crude oil (April et al. 1998; 2000; Colombo et al. 1996; Nwachukwu 2000), often with the help of biosurfactants (Lindley 1992). *Candida utilis* has also been used for the bioremediation of *n*-alkanes in aquatic ecosystems (Nwachukwu 2000).

Methane, the major component of natural gas, is produced by a variety of methanogenic Archaea. It is utilized for growth by methanotrophic yeasts (Wolf and Hanson 1979; 1980) that

lack the ability to grow on methanol, formaldehyde, or formate. Ethane, propane, and *n*-butane, which are also found in natural gas, are oxidized by *Acremonium* sp. and some other fungi to alcohols that can be utilized for growth (Davies et al. 1976).

Straight-chain alkanes, from *n*-decane to *n*-octadecane, are utilized for growth by several yeasts (Ekundayo and Obuekwe 2000; Iida et al. 2000). They are hydroxylated by inducible cytochrome P450 monooxygenases in *C. tropicalis*, *C. maltosa*, and *C. apicola* (Scheller et al. 1996) and *Yarrowia lipolytica* (Iida et al. 2000). Several genes encoding cytochromes P450 have been identified in the alkane-metabolizing yeasts (van den Brink et al. 1998; Iida et al. 2000). *n*-Hexadecane induces conversion of the mycelial form of *Y. lipolytica* to the yeast form (Zinjarde et al. 1998). *Cladosporium resinae* [*H. resinae*], a filamentous fungus which grows on *n*-alkanes, produces an NADH-dependent alkane monooxygenase located in the cytosol (Goswami and Cooney 1999). The insect pathogens *Metarhizium anisopliae* and *Beauveria bassiana* grow on *n*-hexadecane, *n*-tetracosane, *n*-pentacosane, and *n*-octacosane, apparently by β -oxidation (Napolitano and Juárez 1997; Crespo et al. 2000).

The branched alkanes pristane (2,6,10,14-tetramethylpentadecane) and phytane (3,7,11,15-tetramethylhexadecane) are usually considered recalcitrant to microbial attack but can be degraded by several fungi, including *P. boydii* (April et al. 1998; Griffin and Cooney 1979). 3,11-Dimethylnonacosane, another branched alkane, is degraded to shorter-chain hydrocarbons by *M. anisopliae* (Napolitano and Juárez 1997).

Cyclohexane, which occurs in petroleum and is used as a solvent, is transformed cometabolically by several fungi (Griffin and Cooney 1979). A *Cladophialophora* sp. has been

found to make limited growth on cyclohexane (Prenafeta-Boldú et al. 2001). The synthetic compound cyclohexylcyclohexane is cometabolized to a diol by *B. bassiana*, *Cunninghamella blakesleeana*, and *Gongronella lacrispora* (Davies et al. 1986).

Alkenes, produced during the cracking of petroleum and natural gas, have seldom been investigated as possible substrates for fungal degradation. Hexadec-1-ene and heptadec-1-ene are oxidized by *Candida lipolytica* [*Y. lipolytica*] via three different pathways (Figure 1) to epoxides, alcohols, diols, and carboxylic acids (Klug and Markovetz 1968). 1,13-Tetradecadiene is utilized for growth by unidentified fungi (Griffin and Cooney 1979). Cyclohexene is transformed via 2-cyclohexenol to 2-cyclohexenone and 1-methylcyclohexene is transformed via 3-methyl-2-cyclohexenol to 3-methyl-2-cyclohexenone by *Aspergillus cellulosa* [*A. fumigatus*] (Noma et al. 1992).

3 TERPENES

Terpene hydrocarbons (Figure 2) are plant products, synthesized from isoprene units, that are found in the essential oils of plants (Trudgill 1994). Several monoterpenes, sesquiterpenes, and triterpenes are transformed stereoselectively by fungi.

(+)- α -Pinene from oil of turpentine is transformed by *A. niger* to (+)-verbenone, (+)-*cis*-verbenol, and (+)-*trans*-sobrerol (Prema and Bhattacharyya 1962); by *Armillariella mellea* to (+)-*trans*-sobrerol, (+)-*trans*-verbenol, and (+)-verbenone plus seven minor metabolites (Draczyńska et al. 1985); and by *Penicillium* sp. to verbenone (Agrawal and Joseph 2000). (–)- α -Pinene is converted by *A. mellea*

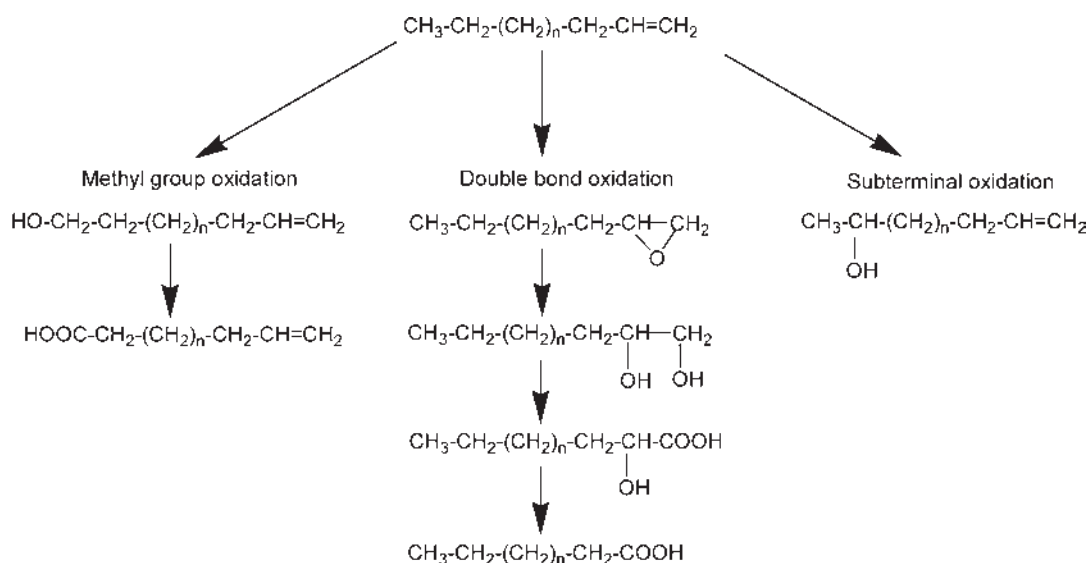


Figure 1 Pathways for the oxidation of 1-alkenes by the yeast *Yarrowia lipolytica* (adapted from Klug and Markovetz 1968).

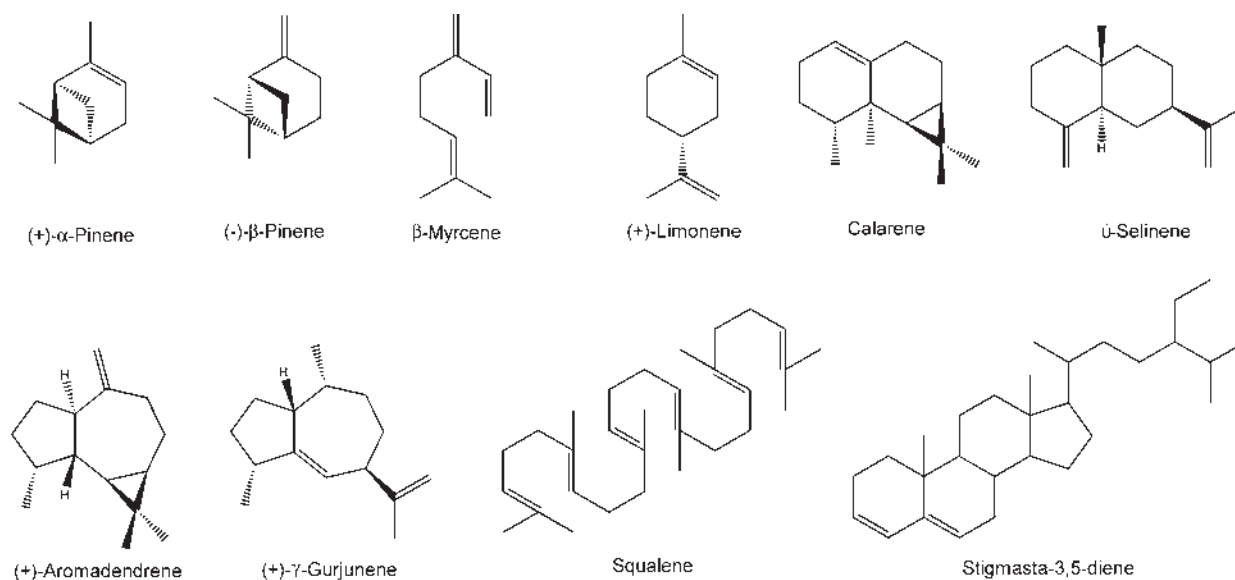


Figure 2 Structures of several terpene hydrocarbons and the steroid hydrocarbon stigmasta-3,5-diene.

to (–)-*trans*-sobrerol, (–)-*verbenone*, (–)-7-hydroxy- α -terpineol, and five other compounds (Draczyńska et al. 1985) and by the yeast *Hormonema* sp. to *trans*-*verbenol* and *verbenone* (van Dyk et al. 1998). (–)- β -Pinene is transformed by *A. mellea* to *trans*-pinocarveol, isopinocamphe, pinocarvone, and six minor metabolites (Draczyńska et al. 1985) and by *Hormonema* sp. to pinocamphe and 3-hydroxypinocamphe (van Dyk et al. 1998).

β -Myrcene, from oil of bay, is transformed by *A. niger*, which adds two hydroxyl groups at any of the double bonds to form three isomeric diols (Yamazaki et al. 1988).

(+)-Limonene, found in orange-peel oil, is transformed by yeasts and filamentous fungi to several products, including (+)-isopiperitenone, (+)-limonene *trans*-1,2-diol, (+)-*cis*-carveol, (+)-perillyl alcohol, (+)-perillic acid, *trans*-isopiperitenol, and α -terpineol (de Oliveira and Strapasson 2000; Noma et al. 1992; van Rensburg et al. 1997; van Dyk et al. 1998). *Penicillium digitatum* converts it mainly to (+)- α -terpineol (Tan et al. 1998). (–)-Limonene is transformed by *A. fumigatus* to (–)-perillyl alcohol, (–)-limonene *trans*-1,2-diol, and (+)-neodihydrocarveol (Noma et al. 1992). *Corynespora cassicola* converts the (+) and (–) isomers of limonene mainly to (+)- and (–)-limonene *trans*-1,2-diol (Demyttenaere et al. 2001).

Several sesquiterpenes (Figure 2) are transformed by fungi. Calarene is oxidized by *Diplodia gossypina* at two of the ring carbons and one of the methyl groups of the cyclopropane ring (Abraham et al. 1992). β -Selinene, (+)-aromadendrene, and its isomer (–)-alloaromadendrene are transformed by *Glomerella cingulata* to triols (Miyazawa et al. 1995; 1997) and (+)- γ -gurjunene is transformed by *G. cingulata* to a diol and a triol (Miyazawa et al. 1998).

The triterpene squalene (Figure 2) and steroid hydrocarbons, such as stigmasta-3,5-diene, are among the lipophilic extractives in eucalyptus wood that have been found to be partially degraded by *Phlebia radiata* and other white-rot fungi (Martínez-Íñigo et al. 2000).

4 MONOCYCLIC AROMATIC HYDROCARBONS

The BTEX compounds (benzene, toluene, ethylbenzene, and the xylene isomers) are toxic components of liquid fuels (Ritchie et al. 2001). They are also commonly used in industry as solvents and chemical feedstocks. Toluene and ethylbenzene support the growth of a few fungi, which have consequently been used for the bioremediation of the volatile components of toxic wastes (Prenafeta-Boldú et al. 2001). Benzene and xylenes do not appear to serve as carbon or energy sources for fungi, although there are reports of the fungal cometabolism of benzene to phenol (Griffin and Cooney 1979) and even of its mineralization to CO₂ (Yadav and Reddy 1993).

Toluene is utilized as a carbon and energy source by *Cladosporium sphaerospermum*, *Scedosporium apiospermum* [the anamorph of *P. boydii*], *Cladophialophora* sp., *Exophiala* sp., and *Pseudeurotium zonatum*; these fungi may be used in biofilters to clean up toluene in waste gases (García-Peña et al. 2001; Prenafeta-Boldú et al. 2001; Weber et al. 1995). In high-nitrogen media, *P. chrysosporium* cometabolizes toluene to CO₂ (Yadav and Reddy 1993).

Ethylbenzene is used as a growth substrate by *C. sphaerospermum*, *Cladophialophora* sp., and *Exophiala*

sp. (Prenafeta-Boldú et al. 2001) and is cometabolized by *P. chrysosporium* (Yadav and Reddy 1993). *Fusarium moniliforme* [*F. verticillioides*] oxidizes ethylbenzene and propylbenzene to (+)-1-phenylethanol and (+)-1-phenylpropanol, respectively (Uzura et al. 2001).

The *o*-, *m*-, and *p*-isomers of xylene are all cometabolized by *P. chrysosporium* but the products are unknown (Yadav and Reddy 1993). Several other dialkylbenzenes, such as 1,4-di-*tert*-butylbenzene, are hydroxylated on the methyl

groups by *Sporotrichum sulfurescens* [*B. bassiana*] (Johnson et al. 1973).

Styrene ($C_6H_5CH=CH_2$) serves as a growth substrate for *Exophiala jeanselmei*, *C. sphaerospermum*, and *Cladophialophora* sp. (Cox et al. 1996; Prenafeta-Boldú et al. 2001). In *E. jeanselmei*, it is oxidized by a cytochrome P450 monooxygenase to styrene oxide, which is converted to phenylacetaldehyde and phenylacetic acid (Cox et al. 1996). *P. ostreatus* oxidizes styrene to

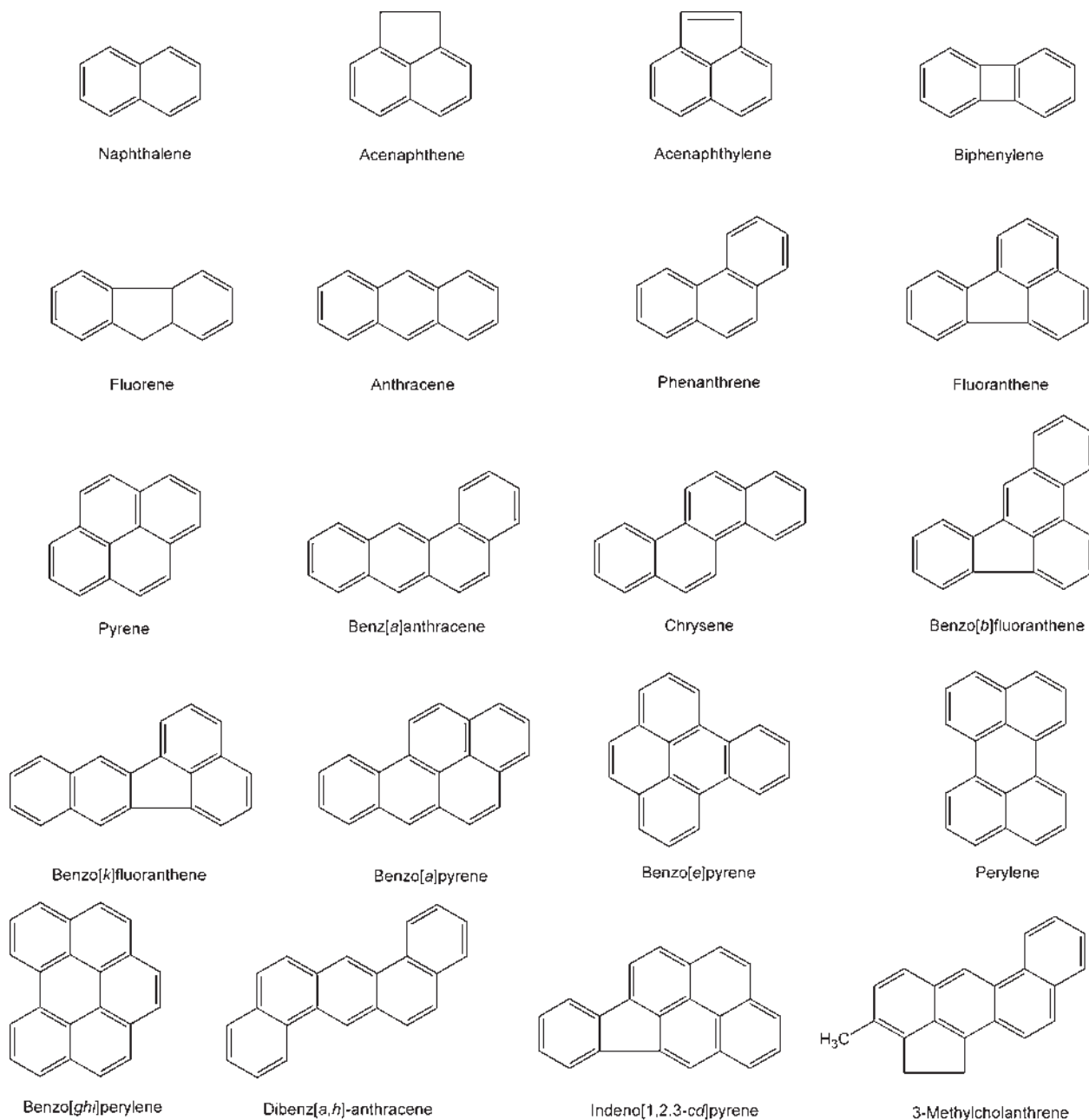


Figure 3 Structures of several polycyclic aromatic hydrocarbons.

phenyl-1,2-ethanediol and other products (Braun-Lüllemann et al. 1997).

5 BIPHENYL

Biphenyl ($C_6H_5C_6H_5$), found in coal tar, oil, and natural gas, is hydroxylated at various positions by *C. elegans* (Dodge et al. 1979). Several yeasts cometabolize biphenyl to 4-hydroxybiphenyl, which *Debaryomyces vanrijae*, *Rhodotorula glutinis*, and *Y. lipolytica* convert further to 4-phenyl-2-pyrone-6-carboxylic acid (Lange et al. 1998; Romero et al. 2001). The yeast *Trichosporon mucoides* cometabolizes biphenyl to 2-, 3-, and 4-hydroxybiphenyl, eight dihydroxylated biphenyls, three trihydroxylated biphenyls, and a quinone (Sietmann et al. 2000; Sietmann et al. 2001). It further transforms the di- and trihydroxylated biphenyls to eight different ring-cleavage products (Sietmann et al. 2001).

6 POLYCYCLIC AROMATIC HYDROCARBONS

The first step in the biotransformation of PAHs (Figure 3) by most fungi is initiated by a cytochrome P450 monooxygenase; white-rot basidiomycetes may also employ lignin peroxidase, manganese peroxidase, or laccase (Hammel 1995; Cerniglia 1997; Cerniglia and Sutherland 2001; Sutherland et al. 1995). Later steps may involve conjugation to form sulfates, glucosides, glucuronides, or xylosides (Casillas et al. 1996). Although PAHs do not generally serve as growth substrates for fungi, the yeast *R. glutinis* is reported to utilize phenanthrene for growth (Romero et al. 1998). The ability of fungi to transform PAHs has been exploited for the bioremediation of contaminated soil (Juhász and Naidu 2000; Cerniglia and Sutherland 2001).

Naphthalene is oxidized by *C. elegans* to 1- and 2-naphthol, naphthalene *trans*-1,2-dihydrodiol, 4-hydroxy-1-tetralone, the 1,2- and 1,4-naphthoquinones, and sulfate and glucuronide conjugates (Cerniglia and Gibson 1977; Cerniglia 1992). Both constitutive and inducible hydroxylases appear to be involved in the pathway (Faber et al. 2001). *A. niger* has been reported (Yogambal and Karegoudar 1997) to metabolize naphthalene via gentisate, a naphthalene metabolite produced by some bacteria. Naphthalene is removed from wastewater by the immobilized mycelia of *P. chrysosporium* (Liao et al. 1997).

Acenaphthene is oxidized by *C. elegans* to 6-hydroxyacenaphthenone, 1,2-acenaphthenedione, acenaphthene *trans*-1,2-dihydrodiol, and four minor metabolites (Pothuluri et al. 1992). The laccase of *Corioloropsis gallica* oxidizes acenaphthene and biphenylene in the presence of an artificial mediator compound, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Pickard et al. 1999), and the laccase of *T. versicolor* oxidizes acenaphthene and acenaphthylene in the presence of 4-hydroxybenzoic acid (Johannes and Majcherczyk 2000).

Fluorene is oxidized by *C. elegans* to 9-hydroxyfluorene, 9-fluorenone, and 2-hydroxy-9-fluorenone (Pothuluri et al. 1993). The first two are also produced by *P. chrysosporium*, *P. ostreatus*, and other fungi (Bezalel et al. 1996c; Bogan et al. 1996; Bumpus 1989; Garon et al. 2000; Schützendübel et al. 1999). The oxidation of fluorene to 9-fluorenone requires manganese peroxidase activity in *P. chrysosporium* and *T. versicolor* (Bogan et al. 1996; Collins and Dobson 1996) but not in *P. ostreatus* or *Bjerkandera adusta* (Schützendübel et al. 1999). The laccases of *C. gallica* and *T. versicolor* are also able to oxidize fluorene (Bressler et al. 2000; Johannes and Majcherczyk 2000).

Anthracene is transformed by *C. elegans* to a *trans*-1,2-dihydrodiol and 1-anthryl sulfate (Cerniglia and Yang 1984); 9,10-anthraquinone has also been reported (Lisowska and Dlugonski 1999). *Rhizoctonia solani* produces the (+) and (−) *trans*-1,2-dihydrodiols and three xyloside conjugates (Sutherland et al. 1992). *Absidia cylindrospora*, *Rhizopus arrhizus*, *Ulocladium chartarum*, *A. niger*, *Cryphonectria parasitica*, and several white-rot fungi also transform anthracene (Field et al. 1996; Giraud et al. 2001; Gramss et al. 1999; Krivobok et al. 1998; Schützendübel et al. 1999; Yogambal and Karegoudar 1997). *P. chrysosporium* degrades anthracene via anthraquinone and phthalate to CO_2 , presumably with the involvement of lignin peroxidase (Hammel et al. 1991). *P. ostreatus* produces anthraquinone and anthracene *trans*-1,2-dihydrodiol (Andersson and Henrysson 1996; Bezalel et al. 1996c; Novotny et al. 1999; Schützendübel et al. 1999); in soil, it incorporates the products into humus (Bogan et al. 1999) or degrades them to CO_2 (Márquez-Rocha et al. 2000). *Pleurotus sajor-caju* [*P. pulmonarius*], *T. versicolor*, and other fungi also produce anthraquinone (Andersson and Henrysson 1996). *Phanerochaete laevis* produces manganese peroxidase and degrades anthracene without accumulating anthraquinone (Bogan and Lamar 1996). The laccases of *C. gallica* and *T. versicolor* oxidize anthracene to anthraquinone (Collins et al. 1996; Johannes and Majcherczyk 2000; Pickard et al. 1999); that of *Nematoloma frowardii*, in the presence of glutathione, can mineralize it (Sack et al. 1997b).

Phenanthrene is oxidized by *C. elegans*, *Syncephalastrum racemosum*, and *A. niger* to *trans*-dihydrodiols, phenanthrols, and sulfate conjugates (Cerniglia and Yang 1984; Casillas et al. 1996; Sutherland et al. 1993). Glucoside and glucuronide conjugates may also be produced (Casillas et al. 1996). *A. niger* is reported to produce 1-methoxyphenanthrene (Sack et al. 1997a) as well as a ring-cleavage product, protocatechuate (Yogambal and Karegoudar 1997). *P. chrysosporium* degrades phenanthrene (Bumpus 1989), producing *trans*-9,10- and 3,4-dihydrodiols, 9-, 3-, and 4-phenanthrols, and a glucoside in high-nitrogen media (Sutherland et al. 1991; Sutherland et al. 1993) and phenanthrene-9,10-quinone and 2,2'-diphenic acid in low-nitrogen media (Hammel et al. 1992). *P. ostreatus* also degrades phenanthrene (Bezalel et al. 1996a; Márquez-Rocha et al. 2000; Schützendübel et al. 1999), producing the *trans*-9,10-dihydrodiol and 2,2'-diphenic acid (Bezalel et al.

1996b) and phenanthrenequinone (Novotny et al. 1999). Several other fungi, including white-rot and ectomycorrhizal fungi, degrade phenanthrene (Colombo et al. 1996; Braun-Lüllemann et al. 1999; Gramss et al. 1999; Field et al. 1996; Lisowska and Długoński 1999; Schützendübel et al. 1999). The manganese peroxidases of *P. laevis* (Bogan and Lamar 1996), *T. versicolor* (Collins and Dobson 1996; Rama et al. 2000), and *N. frowardii* (Sack et al. 1997b) all oxidize phenanthrene. The laccase of *Trametes hirsuta*, in the presence of the mediator 1-hydroxybenzotriazole and linoleic acid, oxidizes phenanthrene via the quinone to 2,2'-diphenic acid (Böhmer et al. 1998); that of *C. gallica*, in the presence of ABTS, also oxidizes it (Pickard et al. 1999). Unlike other fungi, the yeast *R. glutinis* has been reported to utilize phenanthrene as a sole carbon and energy source (Romero et al. 1998).

Fluoranthene is metabolized by *C. elegans* to a *trans*-2,3-dihydrodiol, the 8- and 9-hydroxyfluoranthene *trans*-2,3-dihydrodiols, and two glucoside conjugates (Pothuluri et al. 1990). *A. cylindrospora*, *A. niger*, *U. chartarum*, the yeast *Cryptococcus albidus*, and the white-rot fungi *P. chrysosporium*, *P. ostreatus*, and *B. adusta* also degrade fluoranthene (Liao et al. 1997; Giraud et al. 2001; Gramss et al. 1999; Salicis et al. 1999; Schützendübel et al. 1999). *P. ostreatus* binds fluoranthene metabolites to humus in soil (Bogan et al. 1999).

Pyrene is metabolized to 1-hydroxypyrene, 1,6- and 1,8-pyrenequinones, and three glucoside conjugates by *C. elegans* (Cerniglia et al. 1986). *A. niger* produces 1-methoxypyrene and 1-hydroxypyrene (Sack et al. 1997a) and *P. glabrum* produces these two products plus 1-pyrenyl sulfate, 1,6-dimethoxypyrene, 1,6- and 1,8-dihydroxypyrene, and both quinones (Wunder et al. 1997). *Penicillium janthinellum* and other fungi found in sediments also degrade pyrene (Launen et al. 2000; Ravelet et al. 2000). *P. ostreatus* hydroxylates pyrene to the *trans*-4,5-dihydrodiol and partially mineralizes it (Bezalel et al. 1996a; Bezalel et al. 1996c; Wolter et al. 1997), even in soil (Novotny et al. 1999; Márquez-Rocha et al. 2000). *Crinipellis* sp., *Marasmius* sp., and *Marasmiellus* sp. metabolize it to 1-hydroxypyrene, 1,6- and 1,8-dihydroxypyrene, both quinones, pyrene *trans*-4,5-dihydrodiol, and three sulfate conjugates (Lange et al. 1996). Several other wood-decay and mycorrhizal fungi mineralize pyrene (Braun-Lüllemann et al. 1999; Field et al. 1996; Gramss et al. 1999; in der Wiesche et al. 1996; Schützendübel et al. 1999; Song 1999), as does the manganese peroxidase of *N. frowardii* in the presence of glutathione (Sack et al. 1997b).

Benz[a]anthracene, a carcinogen, is hydroxylated by *C. elegans* to the *trans*-8,9-,10,11-, and 3,4-dihydrodiols and the 8,9,10,11-tetraol (Cerniglia et al. 1994). *P. janthinellum* and the white-rot fungi *P. chrysosporium*, *P. ostreatus*, *P. sajor-caju* [*P. pulmonarius*], and *T. versicolor* also degrade it (Andersson and Henrysson 1996; Wolter et al. 1997; Boonchan et al. 2000). The manganese peroxidase of *P. laevis* degrades it during lipid peroxidation; the 7,12-dione is produced but does not accumulate (Bogan and Lamar 1996).

The manganese peroxidase of *N. frowardii* oxidizes benz[a]anthracene in the presence of glutathione (Sack et al. 1997b).

Chrysene, a weak carcinogen, is metabolized by *C. elegans* to chrysene 2-sulfate and chrysene 2-hydroxy-8-sulfate (Pothuluri et al. 1995) and by *P. janthinellum* to the *trans*-1,2-dihydrodiol (Boonchan et al. 2000; Kiehlmann et al. 1996). *S. racemosum*, *P. ostreatus*, and the ectomycorrhizal fungi *Boletus edulis* and *Amanita muscaria* also degrade chrysene (Braun-Lüllemann et al. 1999; Kiehlmann et al. 1996; Wolter et al. 1997).

Benzo[b]fluoranthene and benzo[k]fluoranthene, which are both carcinogenic, are degraded in soil extracts by *Bjerkandera* sp. (Field et al. 1996) and in wheat straw by *P. ostreatus* (Wolter et al. 1997).

Benzo[a]pyrene, a potent carcinogen, is oxidized by a cytochrome P450 monooxygenase in *C. elegans*, which transforms it to at least 13 compounds (Figure 4) (Cerniglia and Gibson 1979; Cerniglia 1997). The cytochromes P450 of *Aspergillus fumigatus* and other fungi also hydroxylate it (Juhasz and Naidu 2000; Venkateswarlu et al. 1996). Several white-rot and ectomycorrhizal fungi degrade benzo[a]pyrene (Bezalel et al. 1996a; Bumpus 1989; Braun-Lüllemann et al. 1999; Kotterman et al. 1998; Márquez-Rocha et al. 2000; Rama et al. 2000; Wolter et al. 1997; Wunch et al. 1999). *P. chrysosporium* has been used to remove it from water (Liao et al. 1997), and both *P. chrysosporium* and *P. ostreatus* form quinones that can be polymerized in soil (Bogan et al. 1999; May et al. 1997). *P. laevis* produces manganese peroxidase and transforms benzo[a]pyrene to polar products (Bogan and Lamar 1996); the manganese peroxidase of *N. frowardii* can mineralize it in the presence of glutathione (Sack et al. 1997b). The laccases of *T. versicolor*, *Pycnoporus cinnabarinus*, and *C. gallica* oxidize benzo[a]pyrene to quinones in the presence of ABTS (Collins et al. 1996; Pickard et al. 1999; Rama et al. 1998).

Benzo[e]pyrene, a noncarcinogenic isomer of benzo-pyrene, is metabolized by *C. elegans* to sulfate and glucoside conjugates (Pothuluri et al. 1996). Perylene is metabolized by several wood- and straw-degrading fungi (Gramss et al. 1999) and the carcinogenic benzo[ghi]perylene is degraded by *P. ostreatus* (Wolter et al. 1997). Dibenz[a,h]anthracene, a suspected carcinogen, is degraded by the white-rot fungi *P. ostreatus* and *P. sajor-caju* [*P. pulmonarius*] (Andersson and Henrysson 1996; Wolter et al. 1997) and by *P. janthinellum* (Boonchan et al. 2000). Indeno[1,2,3-cd]pyrene, which is also considered carcinogenic, is metabolized by *Bjerkandera* sp. in solvent extracts of contaminated soil (Field et al. 1996). The methylated PAHs, many of which are carcinogenic, can be oxidized to a variety of metabolites. Methyl-naphthalenes, methylanthracenes, methyl- and dimethylbenz[a]anthracenes, and 3-methylcholanthrene are transformed by *C. elegans* (Cerniglia et al. 1982a; 1982b; 1983; 1984; 1990; Wong et al. 1983). In addition, methylanthracenes are transformed by *C. gallica* (Pickard et al. 1999) and methylphenanthrenes by *Fusarium solani* (Colombo et al. 1996).

7 HYDROCARBON DEGRADATION BY MIXED CULTURES

Several experiments have shown that the mineralization of hydrocarbons in nature may require the combined efforts of both fungi and bacteria (Juhasz and Naidu 2000). Pyrene mineralization by *Dichomitus squalens* and *Pleurotus* sp. was

enhanced by soil microorganisms (in der Wiesche et al. 1996). The mineralization of benzo[*a*]pyrene by *Bjerkandera* sp. more than doubled after the addition of activated sludge (Kotterman et al. 1998). *P. janthinellum*, when mixed with the bacterium *Stenotrophomonas maltophilia*, mineralized pyrene, chrysene, benz[*a*]anthracene, benzo[*a*]pyrene, and dibenz[*a,h*]anthracene (Boonchan et al. 2000).

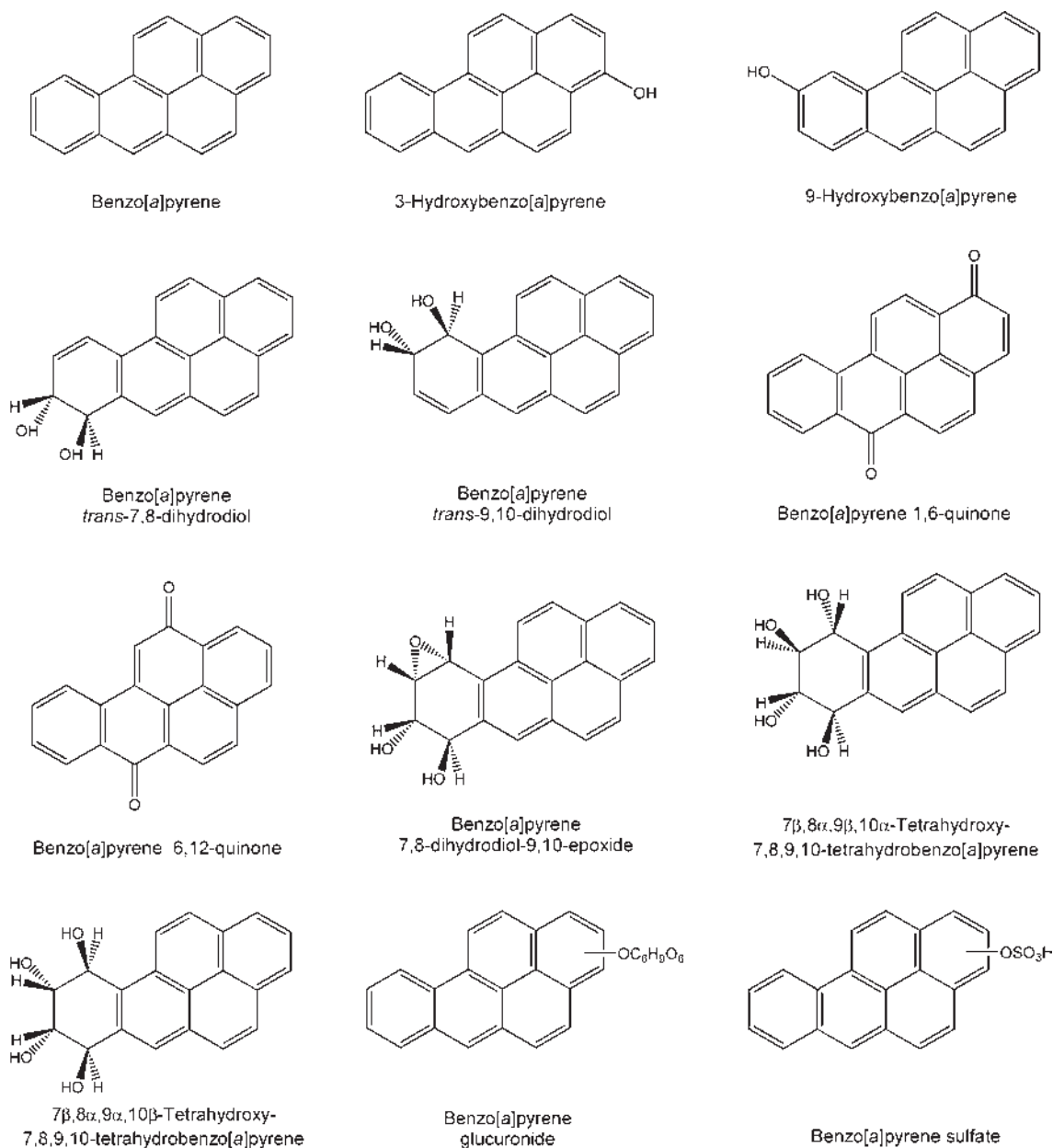


Figure 4 Benzo[*a*]pyrene and the metabolites that have been identified in cultures of *Cunninghamella elegans* (adapted from Cerniglia and Gibson 1979; Cerniglia et al. 1992; Cerniglia 1997). The absolute stereochemistry of some of the compounds and the structures of the glucuronide and sulfate conjugates are not known.

8 BIOTECHNOLOGICAL IMPORTANCE

At one time, most of the industrial research on hydrocarbon metabolism consisted of attempts to produce yeast single-cell protein from petroleum, which was cheap and widely considered to be surplus (Lindley 1992). Little of that type of research is being done now, but there has been extensive progress on the biotransformation of terpenes for the production of flavors, fragrances, and drug intermediates (Trudgill 1994) and on the degradation of PAHs and other toxic hydrocarbons during the bioremediation of soils (Atlas and Cerniglia 1995).

Many of the hydrocarbons contaminating soils and groundwater near leaking fuel tanks, oil spills, and chemical waste dumps can be degraded by fungi (Atlas and Cerniglia 1995; Cerniglia and Sutherland 2001; Colombo et al. 1996). Most of the recent work in this field has been done on PAHs, but some of it has been on BTEX compounds (Yadav and Reddy 1993) and on alkanes (Nwachukwu 2000). Although gigantic oil spills from marine supertankers and offshore oil wells attract immediate public concern, smaller spills from fuel storage tanks are numerous and usually more amenable to bioremediation (Atlas and Cerniglia 1995). The fungal bioremediation of contaminated soils may be accomplished either by removing soil for off-site treatment (May et al. 1997) or by using organic solvents to extract hydrocarbons from the soil for later degradation (Field et al. 1996). On-site bioremediation may be done with fungi that can grow through the soil mass to reach the PAHs (Cerniglia 1997; Novotny et al. 1999). Since few soil microorganisms have the ability to degrade high-molecular-weight PAHs alone (Juhasz and Naidu 2000), fungi and bacteria are now being added together to achieve bioremediation (Boonchan et al. 2000). Fungi have also been used to remove PAHs from wastewater (Liao et al. 1997) and biofilters containing selected fungi have been used to remove toluene and other hydrocarbons from waste gases (García-Peña et al. 2001).

Another application of fungal biotechnology is the transformation of renewable resources, such as the terpenes α -pinene, β -pinene, and limonene, to valuable metabolites that are in demand by the flavor and fragrance industries (Prema and Bhattacharyya 1962; Tan et al. 1998; van Dyk et al. 1998). Fungi usually transform monoterpenes to hydroxylated derivatives and ketones. For instance, (+)-limonene may be hydrated to (+)- α -terpineol, a widely used fragrance chemical (Demyttenaere et al. 2001; Tan et al. 1998), or oxidized to perillyl alcohol, which not only inhibits the growth of some tumor cells but also is a precursor of the artificial sweetener perillaldehyde oxime (de Oliveira and Strapasson 2000). α -Pinene can be oxidized to verbenone, which is used by the flavoring industry (Agrawal and Joseph 2000). Sesquiterpenes are transformed by fungi to a variety of products (Abraham et al. 1992; Miyazawa et al. 1995; 1997; 1998). Other hydrocarbons may be transformed by fungi to intermediates that are useful in the synthesis of pharmaceuticals

and biochemicals (Johnson et al. 1973; Uzura et al. 2001).

9 CONCLUSIONS

Fungi are capable of cometabolizing most hydrocarbons. However, only a few fungi are known to grow on hydrocarbons, such as methane, *n*-hexadecane, toluene, styrene, or phenanthrene, as sole carbon sources. Since various fungi oxidize hydrocarbons, often in a stereoselective manner, some strains have been selected to carry out specific biocatalytic transformations to produce higher-value products. Many fungi transform toxic hydrocarbons to oxidized derivatives and a few strains are able to cleave aromatic rings; thus these fungi have the potential for use, preferably together with bacteria, for the bioremediation of toxic wastes in the environment. Several lines of research on the fungal degradation of hydrocarbons are likely to be productive in the future. These include: (a) Investigating the roles of yeasts and filamentous fungi in transforming naturally occurring hydrocarbons at the concentrations usually found in the environment. (b) Finding additional ways to transform abundant renewable resources, including by-products from agriculture and forestry, into chiral drug precursors and other high-value products. (c) Developing practical combinations of fungi and bacteria that can be used for the large-scale bioremediation of soils contaminated with mixtures of hydrocarbons.

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Biodegradation of Azo Dyes by Fungi

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1 INTRODUCTION

The manufacture and use of dyes and pigments is a multibillion-dollar industry. The use of these substances is an integral part of almost all manufacturing processes. Interestingly, the widespread use of synthetic colorants and the modern dye industry dates only to 1856 with the synthesis of mauveine by W.H. Perkin (Kirk-Othmer Encyclopedia of Chemical Technology 1992). Of all the different types of dyes, azo dyes are the most useful and widely used colorants. In 1858, J. P. Griess synthesized a yellow azo dye that was commercialized briefly (Kirk-Othmer Encyclopedia of Chemical Technology 1992). Other azo dyes that saw early commercialization included Chrysodine (in 1875), Congo Red (in 1884), and Bismark Brown. Following these early syntheses, numerous azo dyes have been made and it is estimated that over 2,000 are in use (Colour Index, 3rd Ed.). Wastewaters are produced during the synthesis and use of dyes. Such wastewaters must be treated. The most important criterion for treatment of wastewater is that associated toxicity is reduced to acceptable levels. Similarly, odors must be reduced and the amount of dissolved material must be reduced. Decolorization of water is also a primary goal of water purification processes and one that is critical to dye-containing wastewaters.

There are several reasons for the popularity and use of azo dyes. Azo dyes are available in almost every color across the spectrum, as a group they are colorfast and many can be structurally modified to bind to a variety of natural and synthetic fabrics (Kirk-Othmer Encyclopedia of Chemical Technology 1992). Unfortunately, azo dyes are resistant to biological degradation. This is likely due to the fact that azo linkages are rarely found in nature. Thus micro-organisms have not often been exposed to compounds containing this functional group and there have been few instances in which

selective pressures on micro-organisms would have favored survival of those species that evolved enzymes capable of specifically destroying azo dyes. The toxicity of azo dyes makes it important that ways be found to treat wastewaters that contain these substances.

This review focuses on the ability of fungi to degrade azo dyes. Before, 1990, there were few reports of the biodegradation of azo dyes by aerobic micro-organisms. Cripps et al. (1990) showed that three azo dyes were extensively degraded by the white rot fungus *Phanerochaete chrysosporium* under aerobic conditions. Since that time, many manuscripts have been published documenting degradation of azo dyes by *P. chrysosporium* and other fungi. Like *P. chrysosporium*, most of the other fungi studied and shown to degrade azo dyes are white rot fungi. Interestingly, however, a few fungi that are not white rot fungi have been reported to have some ability to degrade this important class of compounds. The ability of white rot fungi and other micro-organisms to degrade azo dyes was reviewed by this author several years ago (Bumpus 1995). A number of reviews addressing, at least in part, the biodegradation and/or biodecolorization of azo dyes in water by white rot fungi have appeared recently (Stolz 2001; Fu and Viraraghavan 2001; Knapp et al. 2001; Robinson et al. 2001; McMullan et al. 2001). This review summarizes this area of research and important recent observations while attempting to keep overlap with other reviews at a minimum.

2 DECOLORIZATION AND BIODEGRADATION OF AZO DYES BY WHITE ROT FUNGI

The remarkable biodegradative abilities of the white rot fungus *Phanerochaete chrysosporium* (Bumpus et al. 1985)

led Cripps et al. (1990) to study the ability of this fungus to degrade azo dyes. It was shown that Orange II, Tropaeolin O, and Congo Red were all extensively decolorized in nutrient nitrogen limited cultures of this fungus. Nutrient nitrogen

limited conditions were selected because, in this fungus, such conditions promote expression of the lignin degrading system and it is this system that is thought to be instrumental in biodegradation of xenobiotics. Involvement of the lignin

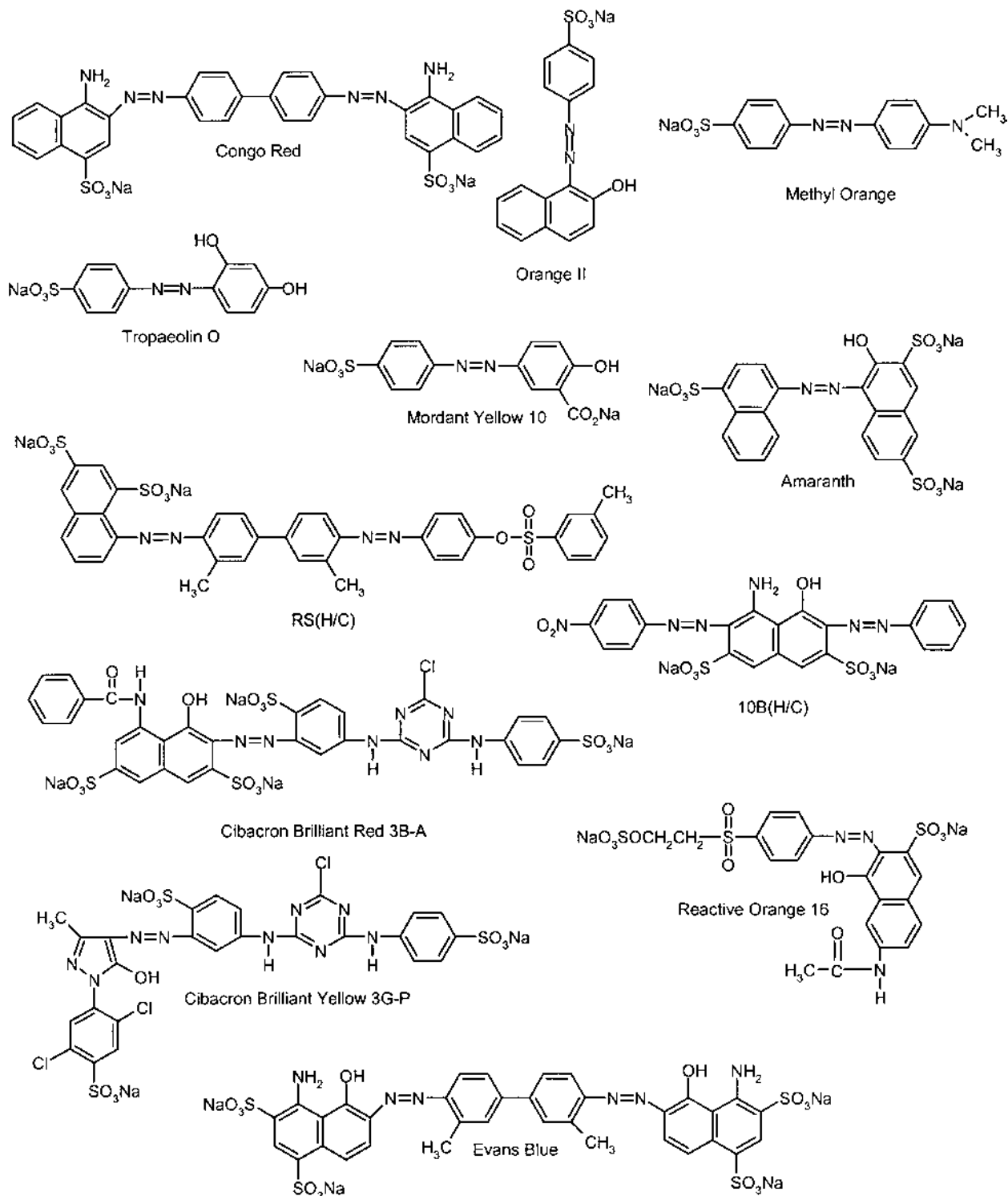


Figure 1 Representative azo dyes degraded by white rot fungi.

degrading system was confirmed by the fact that these dyes were all found to be oxidized by lignin peroxidases. Although initial studies (Cripps et al. 1990) suggested that lignin peroxidases did not oxidize Congo Red, subsequent investigations (Tatarko and Bumpus 1998) showed that this dye is indeed, a substrate for these enzymes.

Decolorization as measured by visible spectroscopy is an exceptionally easy and inexpensive way to monitor degradation of dyes by micro-organisms. However, other techniques are required in order to obtain a true picture of the extent of degradation that occurs. Degradation of azo dyes to carbon dioxide by *P. chrysosporium* was demonstrated by

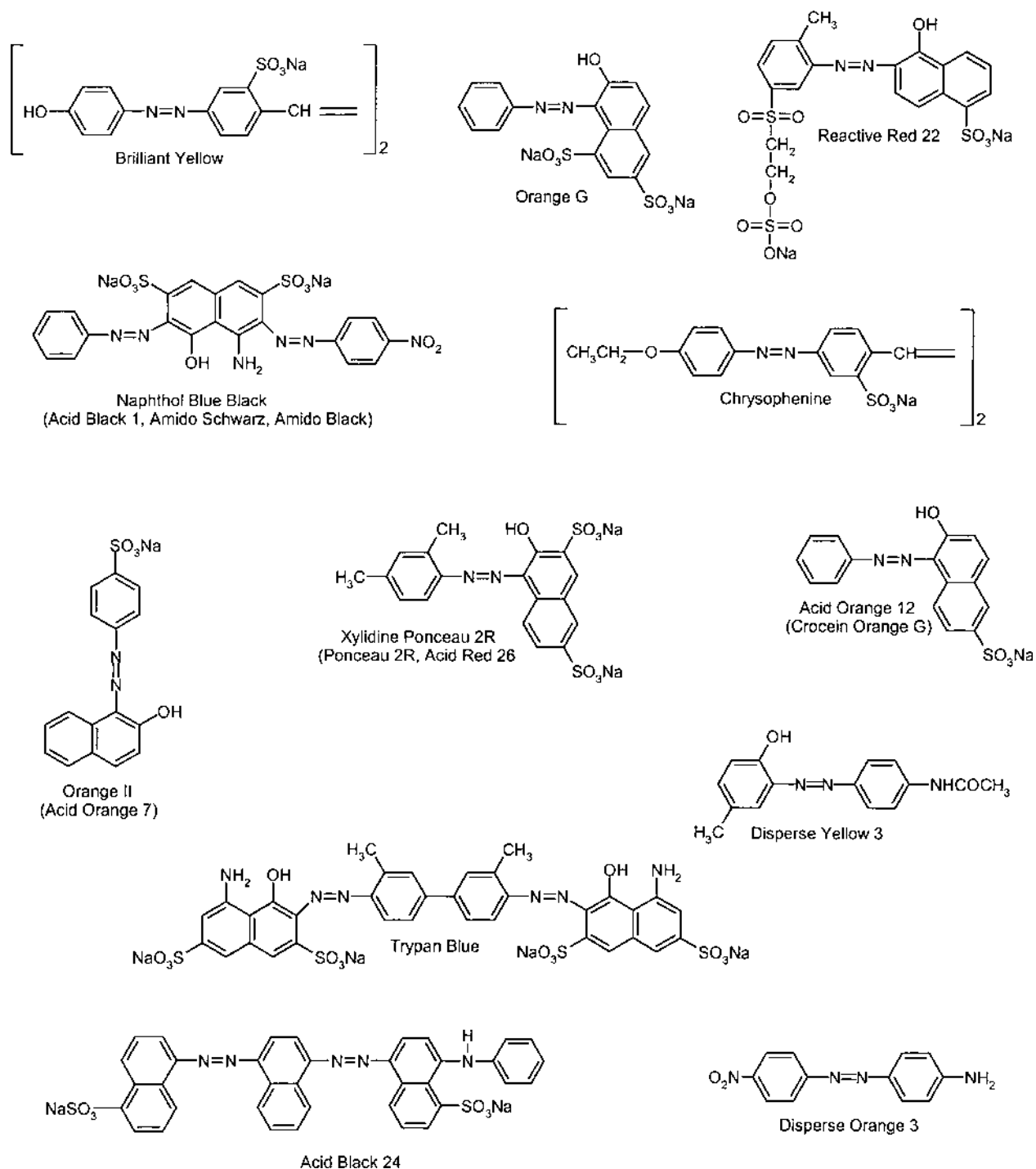


Figure 1 (continued)

Spadaro et al. (1992) who showed that 23.1–48.1% of the aromatic ring labeled carbon atoms in 7 azo dyes (4-phenylazophenol, 4-phenylazo-2-methoxyphenol, 2-(4'-acetamidophenylazo)-4-phenylazophenol (Disperse Yellow 3), 4-phenylazoaniline, N,N-dimethyl-4-phenylazoaniline,

4-(4'-nitrophenylazo)-aniline (Disperse-Orange 3), and 1-phenylazo-2-naphthol (Solvent Yellow 14) were oxidized to carbon dioxide by *P. chrysosporium*. Paszczynski et al. (1992) synthesized several ring-labeled sulfonated azo dyes and showed that substantial quantities (17.2–34.8%) of

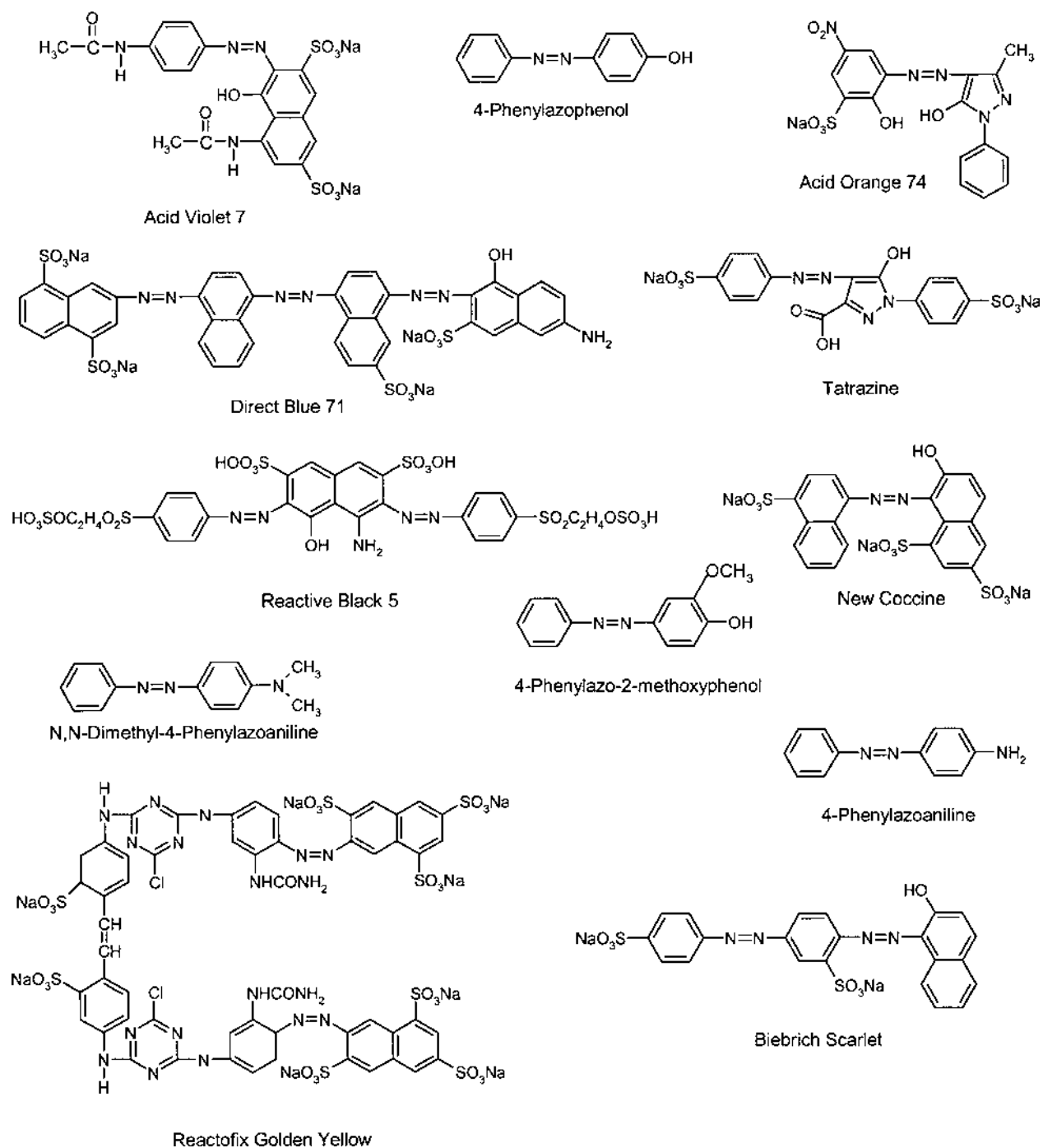


Figure 1 (continued)

the labeled carbon atoms in the dyes were degraded to carbon dioxide by *P. chrysosporium*. Among the dyes degraded to carbon dioxide were Acid Yellow, Orange I, Orange II, 4-(3-methoxy-4-hydroxyphenylazo)benzenesulfonic acid, and 4-(2-sulfa-3'-methoxy-4-hydroxyazobenzenesulfonic acid).

P. chrysosporium has served well as a model microorganism for the study of the degradation of azo dyes and other environmentally persistent organic pollutants. However, it has been shown that a number of other white rot fungi are able to degrade hard-to-degrade compounds, including azo dyes. Representative azo dyes degraded by white rot fungi are illustrated in Figure 1.

As discussed below, some of these fungi have degradative abilities and other properties that will likely make them superior to *P. chrysosporium* for use in bioremediation systems. White rot fungi that have been reported to degrade/decolorize azo dyes are listed in Table 1.

Several *Myrothecium* spp. and *Ganoderma* spp. were shown by Mou et al. (1991) to be able to decolorize Orange II as well as two other diazo sulfonated dyes designated as RS(WC) and IOB(H/C). Several experiments were performed using *M. verrucaria*. Results showed that this fungus was more effective than activated sludge in decolorizing wastewater from a dye-manufacturing plant. Decolorization of wastewater from a textile-dyeing factory was also studied. Five of six highly colored wastewaters were substantially decolorized. It is interesting to note that some experiments were performed at high pH values, one as high as pH 11. In some wastewaters, the concentration of sodium chloride was as high as 15% (w/v). Adsorption to mycelium was shown to be an important mechanism for wastewater decolorization. In some experiments, adsorption was followed by slow decolorization of the mycelium. However, it should be noted that decolorization of mycelium was not observed in all experiments even after extended (1 month) incubation.

The effect of carbon source on the decolorization of water containing Amaranth and Orange G was studied by Chao and Lee (1994). Both dyes as well as the heterocyclic dye Azure B were decolorized by two strains of *P. chrysosporium* and another unidentified white rot fungus. Interestingly, decolorization was reported to occur if fungal strains were pre-cultured in a high nitrogen medium in the presence of glucose. This is of interest as high nitrogen conditions might be expected to repress expression of lignin peroxidases and manganese peroxidases, enzymes that would be expected to mediate decolorization of these dyes.

Knapp et al. (1995) compared the ability of 7 wood-rotting fungi to degrade a variety of dyes, including the azo dyes, Mordant Yellow 10, Cibacron Brilliant Yellow 3G-P, Cibacron Brilliant Red 3B-A, Orange II, Brilliant Yellow, Chrysophenine, Chlorazol Yellow, and Acid Red 106. Results were variable in that all of the fungi were able to degrade extensively some of the dyes. No strain was able to degrade extensively all of the dyes. It is interesting to note that *P. chrysosporium*, was able to mediate 99–100% decolorization of 4 of the 14 dyes and 27–69% decolorization of the other 10. Nevertheless, *P. chrysosporium* was among the least effective of the fungal strains investigated. This study shows clearly that the biodegradative abilities of *P. chrysosporium* are substantial, but it is necessary to assess the biodegradative abilities of other white rot fungi as many will have even greater abilities. These investigations also showed that no one species of fungus was best for all of the dyes studied.

Dye decolorization may be achieved using intact fungal cultures or by using selected fungal enzymes. This may have important implications in bioreactor development. Young and Yu (1997) showed that a variety of dyes, including four azo dyes (Acid Violet 7, Reactive Black 5, Acid Orange 74, and Acid Black 24) were decolorized in culture by *P. chrysosporium* and *Trametes versicolor*. Of interest,

Table 1 Representative manuscripts documenting biodegradation of azo dyes by white rot fungi

Fungus	Dye	Reference
<i>P. chrysosporium</i>	Congo Red, Orange II, Tropaeolin O	Cripps et al. (1990)
<i>Myrothecium</i> spp.	Orange II, RS(H/S), IOB(H/C)	Mou et al. (1991)
<i>T. versicolor</i>	Acid Violet 7, Reactive Black 5, Acid Orange 74, Acid Black 24	Young and Yu (1997)
Strain F29	Orange II	Knapp et al. (1997)
<i>P. ostreatus</i>	Congo Red, Methyl Orange	Shin and Kim (1998)
<i>B. adusta</i>	Reactive Violet 5, Reactive Black 5, Reactive Orange 96, Reactive Red 198	Heinfling et al. (1998a,b)
<i>Bjerkandera</i> BOS55	Amaranth, Remazol Black B, Reactive Blue 15, Remazol Brilliant Orange 3R, Tropaeolin O	Swamy and Ramsay (1999a,b)
<i>T. hirsuta</i>	Amaranth, Remazol Black B	Swamy and Ramsay (1999a,b)
<i>P. sanguineus</i>	Orange G, Amaranth	Pointing and Vrijmoed (2000)
<i>I. lacteus</i>	Congo Red, Methyl Orange, Reactive Orange 16, Reactive Black 5	Novotny et al. (2001)
<i>P. sajorcaju</i>	Amaranth, New Coccine, Orange G	Chagas and Durrant (2001)
<i>F. trogii</i>	Astrazon Red FBL	Yesilada et al. (2002)

however, was the observation that some dyes were more extensively decolorized by a lignin peroxidase preparation from *P. chrysosporium*. For example, 6.6% decolorization of the azo dye Acid Orange 74 was observed in solid agar cultures of *P. chrysosporium*. However, when incubated in the presence of hydrogen peroxide and lignin peroxidase from this fungus, 79.1% decolorization was observed. In some cases, the opposite was observed. In solid agar culture, 98% decolorization of Acid Black 24 occurred whereas when this dye was incubated with lignin peroxidase in the presence of hydrogen peroxide, only 25.4% decolorization occurred.

Shin and Kim (1998) showed that a peroxidase isolated from *Pleurotus ostreatus* decolorized several types of dyes including the azo dyes Congo Red and Methyl Orange. After 4 min, 77 and 26% decolorization of these dyes was noted. Of interest was the observation that addition of veratryl alcohol did not enhance the rate and extent of decolorization. This is in contrast to the observations by Ollikka et al. (1993) for lignin peroxidase from *P. chrysosporium*.

Martins et al. (2001) synthesized several azo dyes having substituent groups [2-methoxyphenol (guaiacol) and 2,6-dimethoxyphenol (syringol)] that would be expected to be found in lignin. Such groups were designated as bioaccessible as they would be expected to be attacked by enzymes from lignin degrading fungi. Not unexpectedly, the extent of dye degradation depended on the concentration of sucrose used as a growth substrate and on the structure of the dye. Interestingly, degradation also depended on the structure of the dye present in cultures of *P. chrysosporium* that were allowed to grow in the presence of the dye and, presumably, the fungus became acclimated to the dye. This is similar to the approach taken by Paszczynski et al. (1991) who synthesized from Acid Yellow 9 and from sulfanilic acid two azo dyes having guaiacol groups. In this study, *P. chrysosporium* degraded these dyes more readily than the parent compounds that lacked the guaiacol group. Interestingly, none of the *Streptomyces* spp. degraded the parent compounds and only 5 of the 9 *Streptomyces* spp. degraded the new guaiacol-linked azo dyes.

Pasti-Grigsby et al. (1992) synthesized 16 azo dyes and assessed their ability, along with 6 commercially available azo dyes (Methyl Orange 52, Ethyl Orange, Acid Yellow 9, Acid Orange 12, Orange 1, and Orange 2), to be degraded by *P. chrysosporium* and by *Streptomyces* spp. *P. chrysosporium* decolorized all of the newly synthesized dyes and the commercially available azo dyes. Most of the newly synthesized dyes underwent substantial (i.e., $\leq 85\%$) decolorization when the initial concentration of dye was 150 mg/L. However decolorization ranged from 27 to 99% at this concentration and from 15 to 97% when the initial concentration was 300 mg/L.

Novotny et al. (2001) studied the biodegradative ability of 103 strains of wood-rotting fungi. A strain of *Irpex lacteus* and a strain of *P. ostreatus* were selected for further investigation to decolorize several different types of dyes including the azo dyes Congo Red, Methyl Orange, Reactive Orange 16, Reactive Black 5, and Naphthol Blue Black. Both

of these fungal strains substantially degraded representative azo and diazo dyes as well as anthraquinone, heterocyclic, triphenylmethane, and phthalocyanine dyes. It is worth mentioning, however, that the diazo dye, Congo Red appeared to be one of the dyes that was comparatively more resistant to degradation. *I. lacteus* caused only 58% decolorization of this dye during 14 days of incubation.

Recently, several other white rot fungi have been shown to be able to degrade azo dyes. Heinfling et al. (1998a,b) showed that *Bjerkandera adusta* is able to degrade azo dyes. These authors have also purified and characterized manganese peroxidases and lignin peroxidases from this fungus and have documented their involvement in azo dye degradation. Azo dyes degraded by *B. adusta* include Reactive Violet 5, Reactive Black 5, Reactive Orange 96, and Reactive Red 198. It is worth noting that *B. adusta* is the most common *Bjerkandera* species in Europe (Heinfling et al. 1998a).

Swamy and Ramsay (1999a,b) studied the ability of *P. chrysosporium*, *Bjerkandera* sp. BOS55, *P. ostreatus*, *Trametes hirsuta* and *T. versicolor* to decolorize 6 dyes, including 4 azo dyes, in solid plate cultures. *P. ostreatus* was not able to decolorize any of the dyes (the azo dyes Amaranth, Remazol Black B, Tropaeolin O, and Remazol Brilliant Orange 3R and the phthalocyanine dye Reactive Blue 15 and the anthraquinone dye Remazol Brilliant Blue R) under investigation. *Bjerkandera* sp. BOS55 and *Trametes versicolor* were most effective in their ability to decolorize these dyes. Both were able to decolorize all of the dyes to some extent. *P. chrysosporium* and *T. hirsuta* possessed intermediate decolorization ability. *P. chrysosporium* did not decolorize Remazol Brilliant Orange 3R and only marginally decolorized Tropaeolin O. This fungus did cause substantial decolorization of Amaranth, Remazol Black B, and Reactive Blue 15. *T. hirsuta* decolorized Amaranth and Remazol Black B. *T. hirsuta* did not decolorize Remazol Brilliant Orange 3R and only marginally decolorized Reactive Blue 15 and Tropaeolin O. It appears that the ability of a given fungus to decolorize a dye depends on certain culture conditions. For example, *P. chrysosporium* did decolorize Remazol Brilliant Orange 3R in solid culture. However, in agitated (200 rpm) pelleted cultures substantial (85% in 18 days) decolorization occurred. Similarly, it was shown that decolorization occurred more rapidly in agitated liquid cultures, in which fungi formed pellets, than in stationary cultures in which the fungi were present as mycelial mats. Although *Bjerkandera* sp. BOS55 was superior in its ability to cause decolorization in solid agar cultures, *T. versicolor* was superior in agitated pelleted liquid cultures. This will be problematic for reactor development because selection of micro-organisms to be used cannot necessarily be based on their performance in solid agar culture. Thus the effectiveness of a micro-organism for dye decolorization will have to be judged on the basis of conditions similar to those under which it will be used in actual wastewater treatment systems. Swamy and Ramsay (1999a,b) also assessed the ability of *Bjerkandera* sp. BOS55, *P. chrysosporium* and *T. versicolor* to decolorize the 5 dyes added sequentially, each after the dye last added had been

completely decolorized. In the last step of this experiment all of the dyes were added as a mixture. In this experiment, only *T. versicolor* was able to degrade all of the dyes. Indeed, *P. chrysosporium* and *Bjerkandera* sp. BOS55 lost their decolorization ability following decolorization of Amaranth and Remazol Black. This investigation demonstrates that the most effective fungal system for decolorization will depend on several things including the choice of fungus, the buffer, the dyes to be degraded, and culture agitation.

Knapp et al. (1997) showed that strain F29, obtained from an unidentified fruiting body from a white rot fungus found growing on rotting willow wood was able to cause substantial (98%) decolorization of water containing up to 1000 mg/L of Orange II in only two days. This is remarkable as Pasti-Grigsby et al. (1992) showed that it took *P. chrysosporium* 15 days to achieve 53 and 99% decolorization of water containing 300 and 150 mg/L Orange II, respectively. Knapp et al. (1997) reported that a carbon source other than Orange II was required for decolorization and that a small amount of nutrient nitrogen (0.25 g dm^{-1}) seemed optimal and necessary to maintain decolorization ability in long-term experiments. A pH of 5.3–6.3 appeared to be optimal. Addition of veratryl alcohol provided a slight improvement in the rate of decolorization. However, addition of Mn(II) resulted in a decolorization rate 70% greater than that of controls to which no additional Mn(II) was added. These results led this research group to use this fungus in the development of bioreactors (Zhang et al. 1998) as discussed elsewhere in this review.

Pointing and Vrijmoed (2000) studied the ability of *Pycnoporus sanguineus* to decolorize two triphenylmethane dyes (Bromophenol Blue and Malachite Green) and two azo dyes (Orange G and Amaranth). These investigators also found that the azo dyes were more resistant to degradation than the other dyes studied. Complete or nearly complete decolorization of the triphenylmethane dyes occurred while only partial (up to 60%) decolorization of the azo dyes was observed. Adsorption of dye to mycelium was not a major factor in decolorization by this fungus.

Yesilada et al. (2002) showed that *Funalia trogii* in pelleted form could decolorize relatively high concentrations (264 mg/L) of Astrazon Red FBL. Astrazon dyes are widely used basic dyes. In these investigations, which were the first to study biodegradation of Astrazon dyes by a white rot fungus, it was shown that at a dye concentration of 13 mg/L,

fungal pellets could be reused up to five times with great effectiveness (i.e., 92–98% color removal occurred). At higher concentrations (264 mg/L) similar color removal was observed only when an increased amount of fungal mass was used. However, in these experiments, repeated use of the fungal pellets resulted in lower amounts (0–73%) of decolorization. It appears that the dye is toxic to the fungus at high concentrations. Nevertheless, it is clear that fungal pellets can be reused several times to remediate water containing dyes at concentrations that might be expected to be present in wastewater. Also of importance was the observation that dye degradation occurred over the pH range 6–11. It should also be noted that, like several other white rot fungi, dye adsorption by fungal mycelium was followed by decolorization of the mycelium.

Chagas and Durrant (2001) compared the ability of *P. chrysosporium* and *Pleurotus sajorcaju* to decolorize four azo dyes. *P. chrysosporium* in solid culture medium partially decolorized Amaranth, New Coccine, Orange G, and Tartrazine. *P. sajorcaju* decolorized Amaranth, New Coccine, and Orange G. It did not decolorize Tartrazine. In liquid cultures of *P. chrysosporium* total decolorization of Amaranth, New Coccine, and Orange G and 60% decolorization of Tartrazine was observed. *P. sajorcaju* mediated total decolorization of Amaranth and Coccine, 50% of Orange G and 20% of Tartrazine. In these investigations, lignin peroxidase activity was not observed. However, manganese peroxidase activity was observed in cultures of *P. chrysosporium* and laccase activity was found in cultures of *P. sajorcaju* suggesting that these enzymes are important in dye degradation.

3 DECOLORIZATION AND BIODEGRADATION OF AZO DYES BY FUNGI OTHER THAN WHITE ROT FUNGI

In recent years there have been many reports regarding biodegradation of azo dyes by white rot fungi (Bumpus 1995; Stolz 2001; Yuzhu and Viraraghavan 2001; Knapp et al. 2001; Robinson et al. 2001; McMullan et al. 2001). In contrast, there have been only a few reports focusing on biodegradation and/or decolorization of azo dyes by fungi that are not white rot fungi. Some of these fungi are listed in Table 2.

Table 2 Manuscripts describing adsorption of or biodegradation of azo dyes by fungi other than white rot fungi

Fungus	Dye	Reference
<i>N. crassa</i>	Vermelho Reanil P8B	Corso et al. (1981)
<i>A. sojae B-10</i>	Amaranth, Sudan II, Congo Red	Behung-Ho and Weon (1992)
<i>A. foetidus</i>	Drimarene Red, Drimarene Blue	Sumanthi and Phatak (1999)
	Drimarene Black	Sumanthi and Manju (2000)
<i>C. inaequalis</i>	Chicago Sky Blue 6B	ten Brink et al. (2000)

As noted by Chung and Stevens (1993) an early report by Riedel (1942) showed that azo dyes were reduced by yeasts but that pure culture isolations were not reported in this study. In a study focusing on the use of fungi to treat industrial effluents containing an azo dye it was shown by Corso et al. (1981) that the Ascomycete *Neurospora crassa* was able to decolorize water containing Vermelho Reanil P8B. In these studies 91.3–89.1% decolorization was achieved during a 24-hr incubation period in water in which the dye concentration was 16–32 mg/L. These dye concentrations are typical of those that would be found in wastewater. Behung-ho and Weon (1992) reported that the Ascomycete *Aspergillus sojae* B-10 was able to mediate substantial decolorization of Amaranth, Sudan III, and Congo Red. The effect of several nutrient nitrogen sources on decolorization was assessed. Nitrogen containing salts (ammonium tartrate, sodium nitrate, ammonium nitrate, and ammonium sulfate) appeared to promote decolorization relative to biologically derived nitrogen sources (peptone, malt extract, and yeast extract). It was also shown that decolorization decreased as a function of sodium nitrate concentration for all three dyes that were studied. Adsorption contributed to the decolorization that was observed. However, the authors assert that biodegradation was also important in this process. If this decolorization is, indeed, due to biodegradation and not simple adsorption it will be interesting to determine the mechanisms by which biodegradation occurs as they will almost certainly be different than those which occur in white rot fungi.

Sumanthi and Phatak (1999) and Sumanthi and Manju (2000) showed that *Aspergillus foetidus* was able to decolorize in culture several azo dyes (Remazol Red, Remazol Dark Blue HR, Remazol Brown GK, Drimarene Red, Drimarene Blue, Drimarene Black, Procion Green, and Procion Turquoise). However, these investigators noted that decolorization was due primarily to biosorption and that little biotransformation occurred.

Curvularia spp. are members of the Ascomycetes and include animal and plant pathogens. Unlike the other fungi discussed in this review, *Curvularia inaequalis* produces a peroxidase that contains vanadate rather than heme (ten Brink et al. 2000). This vanadium containing peroxidase requires hydrogen peroxide as a cosubstrate and mediates oxidation of halides to hypohalous acid. It also mediates the sulfoxidation of organic sulfides to sulfoxides and the one-electron oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to its free radical product (ten Brink et al. 2000). It has been shown that the vanadate-containing peroxidase from this fungus mediates oxidation of the azo dye Chicago Sky Blue 6B (ten Brink et al. 2000).

4 SELECTED ASPECTS OF THE ENZYMOLGY OF AZO DYE DEGRADATION BY WHITE ROT FUNGI

White rot fungi are so named because they are able to preferentially degrade lignin in wood. When this occurs in

nature substantial amounts of cellulose often remain giving the decayed wood a white appearance. Conversely when brown rot fungi attack wood residual lignin, which is brown in color, remains. Thus one of the consequences of biodegradation by white rot fungi is decolorization of naturally occurring substances. The enzymes responsible for the initial stages of biodegradation and decolorization are oxidative enzymes, primarily lignin peroxidases (ligninases), manganese peroxidases, and laccases (Kirk and Farrell 1987). Lignin peroxidases and manganese peroxidases have reaction mechanisms similar to those of other peroxidases (e.g., horseradish peroxidases and lactoperoxidase) (Dunford 1999). These peroxidases contain a heme prosthetic group and require hydrogen peroxide as an oxidizing cosubstrate. In the first step of the peroxidase reaction mechanism the enzyme undergoes a two-electron oxidation in which it is converted to compound I. Compound I is an activated form of the enzyme in which the heme iron exists as an oxyferryl species in the 4 + oxidation state. The other equivalent exists as a porphyrin cation radical or, in some cases, as another radical species. The oxygen atom in the oxyferryl species is donated by hydrogen peroxide. The other oxygen atom originally present in hydrogen peroxide is reduced to water. Compound I is a good oxidant and is able to mediate the one-electron oxidation of a variety of organic pollutants, including several azo dyes. During the one-electron oxidations mediated by compound I, this activated peroxidase intermediate is reduced to compound II, which is also an activated oxyferryl peroxidase intermediate. Unlike compound I, which has an oxidation state that is two electron equivalents greater than the ferric resting state, the oxidation state of compound II is one electron equivalent greater than the ferric resting state. Thus another one-electron oxidation of an azo dye or other reducing substrate converts compound II back to the resting state thereby completing the reaction cycle.

Lignin peroxidases and manganese peroxidases are relatively nonspecific in that they are able to oxidize a variety of substrates. On the other hand, these enzymes also exhibit some specificity. This has been demonstrated nicely by Pasti-Grigsby et al. (1992) who showed that some azo dyes are preferentially oxidized by lignin peroxidases whereas others are preferentially oxidized by manganese peroxidases.

Veratryl alcohol is an endogenous substrate for lignin peroxidases produced by *Phanerochaete chrysosporium*. When this compound serves as substrate it undergoes two successive one-electron oxidations to form veratraldehyde. Of interest to this review is the fact that veratryl alcohol enhances oxidation of some substrates, including some azo dyes. It appears that some azo dyes (e.g., Biebrich Scarlet and Tartrazine) can undergo a one-electron oxidation by lignin peroxidase compound I forming the one-electron oxidation product of the dye and lignin peroxidase compound II (Paszyzyski and Crawford 1991). However, compound II does not appear to be able to mediate oxidation of the azo dye. This results in accumulation of lignin peroxidase compound II and the reaction ceases. When veratryl alcohol is added, compound II oxidizes it and returns to the ferric resting state

of the enzyme which may then participate in another round of catalysis. By preventing accumulation of compound II veratryl alcohol enhances oxidation of the azo dye.

Wu et al. (1996) showed that nutrient carbon or nutrient nitrogen limited cultures of *P. chrysosporium* are able to decolorize Reactive Red 22. It was also reported that a crude preparation of lignin peroxidases mediated decolorization of Reactive Red 22 and another azo dye, Evan's Blue. Similar to the observations of Paszyzyski and Crawford (1991) it was found that the presence of veratryl alcohol enhanced decolorization of Reactive Red 22.

Manganese peroxidases are so named because they mediate the one-electron oxidation of Mn(II) to Mn(III) (Kuwahara et al. 1984; Glenn et al. 1986; Wariishi et al. 1988). *In vitro*, this reaction is often performed in the presence of a metal chelator such as lactate or tartrate, the purpose of which is to facilitate dissociation of Mn(III) from the enzyme in the form of a Mn(III)-lactate or Mn(III)-tartrate complex (Wariishi et al. 1992).

In vivo, oxalate or malonate likely performs this function. This is of interest because Mn(III)-complexes are relatively good oxidants. They are also relatively stable. It is thought that Mn(III)-complexes are stable enough to diffuse away from the active site and mediate oxidation of lignin and other materials that are inaccessible or otherwise not amenable to direct oxidation by laccases, lignin peroxidases, or manganese peroxidases. Recent studies have shown that the specificity of manganese peroxidases varies considerably between fungal species. For example, Heinfling et al. (1998a,b) showed that Reactive Orange 96 and Reactive Red 198 were not oxidized by manganese peroxidase P1 from *P. chrysosporium* and that oxidation of Reactive Blue 5 by this enzyme was negligible. This enzyme slowly oxidized Reactive Violet 5 and activity was increased by approximately five-fold when 0.3 mM MnSO₄ was present in the reaction mixture. In contrast, the presence of 0.3 mM MnSO₄ did not enhance oxidation of the other azo dyes studied. The ability of manganese peroxidases from other white rot fungi to oxidize these azo dyes was also studied. Of importance is the observation that substantial rates of dye oxidation occurred in reactions that were independent of manganese. Indeed, Mn(II) was shown to function as a noncompetitive inhibitor of Reactive Black 5 oxidation by MnP1 from *B. adusta*. Manganese peroxidases from these fungi allow a strategy of direct oxidation (i.e., by the enzyme) or indirect oxidation (by Mn(III)-complexes) to be pursued. This may become important in bioreactor design and strategy development.

In addition to lignin peroxidases and manganese peroxidases, white rot fungi often produce laccases. These oxidases are also important in lignin degradation but unlike peroxidases, they do not contain heme. Laccases are more formally known as benzenediol:O₂ oxidoreductases, EC 1.10.3.2). Instead of heme, laccases require active site Cu(II) ions for activity. During laccase-mediated reactions, diphenolic compounds undergo a four-electron oxidation. During this reaction, Cu(II) is reduced to Cu(I). During the next step in the reaction, Cu(I) reduces molecular oxygen (O₂) to

produce two molecules of water. During this reaction Cu(I) is oxidized back to Cu(II) thus completing the reaction cycle. Laccases can mediate other reactions. Wong and Yu (1999) showed that *Trametes versicolor* decolorized Acid Violet 7. Of interest was the observation that laccase did not directly oxidize this azo dye. However, in the presence of a low molecular weight compound substantial oxidation (i.e., decolorization) of this dye occurred. It was proposed that the low molecular weight compound was oxidized by laccase producing a reactive radical species, which then oxidized the dye molecule resulting in a colorless product.

Pointing and Vrijmoed (2000) have presented evidence implicating laccases produced by *Pycnoporus sanguineus* in the oxidation of the azo dyes Orange G and Amaranth and the triphenylmethane dyes Bromophenol Blue and Malachite Green. Evidence for this, however, was indirect as oxidation of these dyes was only correlated with laccase activity in cultures of this fungus. It should be noted, however, that *P. sanguineus* did not produce lignin peroxidase or manganese peroxidase.

Yesilada and Ozcan (1998) studied the ability of crude culture filtrates to decolorize Orange II. It was shown that filtrates from *Coriolus versicolor* but not *P. chrysosporium*, were able to mediate substantial decolorization of water contaminated with this dye. Decolorization was dependent on the age of the culture and was not dependent on the presence of hydrogen peroxide. Decolorization activity was inactivated by heat. Given the fact that *C. versicolor* produces laccase in abundance it is reasonable to suggest that this enzyme is responsible for the decolorization observed.

Production of veratryl alcohol radical and Mn(III) complexes may be of importance in lignin degradation as it has been suggested that these low molecular weight reactive species may be stable enough to diffuse into the 3-D lignin structure and mediate oxidations that are not accessible by direct enzymatic oxidation by lignin peroxidases, manganese peroxidases, or laccases (Barr and Aust 1994; Harvey et al. 1986; Glenn et al. 1986). Veratryl alcohol radical may be too unstable and short-lived to accomplish this. However, Mn(III) complexes do appear to be sufficiently stable for this purpose. Mn(III) complexes appear to be able to oxidize phenolic units in lignin. They do not appear to be able to oxidize nonphenolic units (Popp and Kirk 1992; Wariishi et al. 1992). However, when unsaturated fatty acids are added to reaction mixtures containing manganese peroxidase, Mn(II) complexes, and hydrogen peroxide, lipid peroxidation occurs (Kapich et al. 1999). Of interest is the observation that oxidants are produced, in such reaction mixtures, which are capable of oxidizing nonphenolic subunits in lignin as well as selected organic pollutants such as phenanthrene. Kapich et al. (1999) suggest that peroxy radicals are produced that are responsible for the oxidations that occur.

Lignin peroxidases, manganese peroxidases, and laccases are by far the most studied oxidases found in white rot fungi. Because of their ability to degrade a wide range of organic compounds, it was reasonable to suspect that cytochrome P-450 monooxygenases may be responsible for some of

the oxidations that have been observed. Indeed, some of the documented oxidations that are mediated by *P. chrysosporium* have not been attributed to lignin peroxidases, manganese peroxidases, or laccases. As a case in point, during DDT degradation, the parent compound undergoes hydroxylation, forming dicofol (Bumpus et al. 1985). To date, the enzyme responsible for this oxidation has not been identified and may well, indeed, be a cytochrome P-450 monooxygenase. This family of enzymes has not been extensively investigated in white rot fungi. Studies by Knapp et al. (1997) and by Yadav and Loper (2000) showed that P-450 monooxygenase genes exist in this fungus. Substantial evidence for P-450 mediated hydroxylation of benzo(a)pyrene was presented by Masaphy et al. (1996) who showed that microsomal and soluble fractions from *P. chrysosporium* exhibited characteristic reduced carbon monoxide difference spectra. Benzo(a)pyrene hydroxylation was shown to be dependent on NADPH and was inhibited by carbon monoxide. Furthermore benzo(a)pyrene caused a type I spectral shift (indicative of substrate binding) when added to soluble and microsomal preparations. A cytochrome P-450 monooxygenase is also thought to be responsible for phenanthrene oxidation during biodegradation of this compound by *Pleurotus ostreatus* (Bezalele et al. 1996).

Most recently, the genome of *P. chrysosporium* has been sequenced at the U.S. Department of Energy's facility at Walnut Hill, California. This fungus is the first basidiomycete whose genome has been sequenced. The genome is about 30 Mb and is comprised of 10 chromosomes. Of interest is the observation that this fungus may have the genetic potential to produce over one-hundred P-450 monooxygenases (Nelson 2001). Clearly, elucidation of the contribution of P-450 monooxygenases to azo dye degradation and xenobiotic metabolism, in general, will be an area of considerable research interest.

5 METAL IONS AND THE POSSIBLE ROLE OF SIDEROPHORES IN BIODEGRADATION OF DYES

Kirk et al. (1986) developed culture media for *Phanerochaete chrysosporium* containing certain supplemental nutrient mixtures of metal salts that enhance the level of lignin peroxidase activity produced in cultures of this fungus. Pointing et al. (2000) studied the ability of *P. chrysosporium* (IMI 284010), *Pycnoporus sanguineus* (HKUCC 4065), *Trametes versicolor* (HKUCC 4063), and an unidentified fungal strain (HKUCC 4062) to decolorize several azo, triphenylmethane, heterocyclic, and polymeric dyes. All four strains were described as sub-tropical basidiomyceteous fungi. *P. chrysosporium*, *T. versicolor*, and the unidentified strain completely decolorized in liquid cultures the azo dyes Amaranth, Amido Black, Aniline Blue, Congo Red, Methyl Orange, Orange G, Ponceau 2R, and Trypan Blue during a 14-day incubation period. *P. sanguineus* decolorized Amido Black, Aniline Blue, Methyl Orange, Orange G, and Trypan

Blue. This fungus did not decolorize Amaranth, Congo Red, and Ponceau 2R. These investigators also studied the ability of these fungi to decolorize several triphenylmethane, heterocyclic, and polymeric dyes. Several dyes were decolorized. However, in general, the azo dyes appeared to be more resistant to degradation. Also studied were the inhibitory effects of three metal ions [Cd(II), Cu(II), and Zn(II)] on dye degradation. These studies were performed using Poly R 478. It was shown that *T. versicolor* (HKUCC) and the unidentified strain (HKUCC 4062) decolorized this dye in the presence of 0.1 mM Cu(II), and Zn(II). Cd(II) appeared inhibitory to *T. versicolor* at this concentration. Interestingly, the unidentified strain was able to mediate decolorization of Poly R 478 at a Cd(II) concentration of 0.25 mM. In contrast, when *P. chrysosporium* was studied, decolorization was inhibited by metal ion concentrations of 0.1 mM. Although Poly R 478 is not an azo dye, this study suggests that the presence of metal ions in dye containing wastewater is one of the factors that must be taken into consideration in developing bioreactors based on the use of basidiomyceteous fungi.

Fungi often grow in conditions where iron concentrations are vanishingly low. Iron is essential for metabolism (i.e., electron transport system). Thus fungi have evolved ways of sequestering this essential element. To do this, fungi secrete siderophores having very high affinities for iron and certain other polyvalent cations and it is in the form of these metal siderophore complexes that metals are absorbed by fungi (Fekete et al. 1989). In addition to their role in sequestering metals, siderophores may have another important role as some siderophore-metal complexes appear to exhibit phenoloxidase-like activity. Some siderophore iron complexes are reduced from the ferric (III) to the ferrous(II) oxidation state. When lignin is the electron donor, measurable lignin degradation takes place. Minussi et al. (2001) have studied this phenomenon using several dyes and have presented circumstantial evidence that siderophore-metal complexes might be involved in the decolorization of Reactive Blue 19, Reactive Red 195, Reactive Yellow 145, and Reactive Black 5 by wood rotting fungi. Although these studies are inconclusive, the involvement of metal-siderophore complexes in the decolorization of azo dyes is an area that deserves further scrutiny.

6 DEVELOPMENT OF BIOREACTORS USED FOR REMEDIATION OF AZO DYE CONTAINING WASTEWATER

White rot fungi have been proposed for remediation of soils contaminated with toxic organic pollutants such as DDT, polychlorinated phenols, and polycyclic aromatic hydrocarbons (Bumpus 1993). However, decolorization and remediation of wastewater from a variety of industries that produce colored wastewater may be the most promising place for use of these fungi in bioremediation systems. Processes

developed to remediate colored wastewaters have been the subject of considerable research that has been reviewed recently by Knapp et al. (2001). Although many investigations have focused on the ability of white rot fungi to decolorize/degrade azo dyes, only a few investigations have specifically focused on development of bioreactors that could be scaled up and used for this purpose. Zhang et al. (1998) showed that an unidentified basidiomycete designated F29 was able to extensively and rapidly decolorize high concentrations of the azo dye Orange II in three different bioreactor configurations. Continuous packed-bed, fedbatch fluidized-bed, and continuous fluidized-bed bioreactors were studied. All of the bioreactors studied proved suitable for bioremediation of water-containing Orange II. Concentrations of dye up to 1000 mg/L were removed relatively rapidly (1–3.5 days) and the mycelium could be used repeatedly. Particular success was achieved with the fedbatch fluidized bed-immobilized reactor. In this system average decolorization rates of $40\text{--}50\text{ mg l}^{-1}\text{ hr}^{-1}$ were achieved resulting in 97% color removal in 24 hr. This study is significant as it demonstrates that rapid degradation occurs and the fungal mycelium are robust and unaffected by exposure to high concentrations of Orange II during repeated exposure over 1–2 months.

Swamy and Ramsay (1999a,b) studied conditions, which promote azo dye degradation by *Trametes versicolor*, *Bjerkundera adusta* and *Phanerochaete chrysosporium* sequentially in batch reactors. Only *T. versicolor* proved to be able to maintain its ability to cause decolorization of water following repeated addition of the several dyes and dye mixtures.

Wang and Yu (1998) studied the ability of *T. versicolor* to decolorize water containing an anthroquinone dye, an indigo dye, and the azo dye Acid Violet 7. All of the dyes were degraded. These investigators also showed that adsorption to mycelium followed by dye degradation occurred. Adsorption was rapid and occurred with living mycelium and with heat killed controls. With live fungi, mycelium was regenerated by physical desorption and enzymatic degradation. The authors suggest that a sequential treatment system could be developed in which dyes in wastewater could be first adsorbed on fungal mycelium followed by decolorization/degradation. It is reasonable to suggest that sequencing batch reactor technology might function well in this situation. Indeed, Borchert and Libra (2001) have used *T. versicolor* in 4-L sequencing batch stirred tank reactors to decolorize water containing the azo dyes Reactive Black 5 and Reactive Red 198 and the anthroquinone dye Brilliant Blue R. Reactors were cycled repeatedly over the course of the experiments. During long-term (200 days) experiments under sterile conditions, 18 dye additions occurred. In each case substantial color removal occurred. When the initial concentration of dye was 100 mg/L, 97–99.5% decolorization occurred. When the initial concentration was 500 mg/L, 91–99% decolorization occurred. When similar experiments were performed under nonsterile conditions, only 5 decolorization cycles (during a 55-day experiment) were possible due to bacterial

contamination, which appeared to interfere with peroxidase activity, resulting in decreased decolorization. Biodegradation of Acid Violet 7 was also studied in liquid batch cultures and in a fluidized bed reactor (Zhang and Yu 2000). In both cases, the ability of mycelial pellets to degrade this dye was studied. An interesting aspect of this research was that added activated charcoal had a positive effect on biodegradation. It was shown that fungal mycelium surrounded a core of activated charcoal to form complex pellets. These complex pellets appeared to enhance biodegradation by adsorbing both the dye and fungal enzymes responsible for biodegradation. Furthermore, decolorization by the complex pellets was superior to that observed for fungal pellets without activated charcoal, for fungal pellets to which activated charcoal was added (after pellet formation) and to activated charcoal alone. It was also shown that reactors operated in repeated-batch-fed mode resulted in greater and more efficient decolorization than reactors operated in a continuous flow mode. In one experiment a repeated-batch-fed reactor was able to decolorize 9 additions of dye over a period of 130 h. Batch-fed additions of dye ranged from 100 to 500 mg/L.

Mielgo et al. (2001) studied the use of immobilized *P. chrysosporium* in continuous flow packed bed bioreactors. At dye loading rates of $0.2\text{ g l}^{-1}\text{ d}^{-1}$ greater than 95% decolorization was achieved at hydraulic retention times of 24 h. In these experiments, the temperature was maintained at 37°C and oxygen was supplied in a pulsed flow.

Trametes hirsuta was shown to effectively decolorize the azo dyes Reactive Black 5 and Direct Blue 71 as well as representative triphenylmethane, indigoid, and anthraquinone dyes (Abadulla et al. 2000). All of the dyes were decolorized by the laccase isolated from *T. hirsuta*. This research is important as it addressed the issue of recycling dye-contaminated water. Water decolorized using the intact fungus or laccase in solution was not suitable for reuse in dyeing operations due, presumably, to interference by soluble protein. However, when a reactor containing immobilized laccase was used, the recycled decolorized wastewater was suitable for dyeing operations as the laccase was retained in the bioreactor.

7 CONCLUSIONS

It is clear that white rot fungi are able to mediate extensive and often rapid degradation of azo dyes. Although several micro-organisms (other fungi and some bacteria) have been reported to be able to degrade this class of pollutant, it appears that white rot fungi have superior biodegradative abilities in this regard. The enzymes (lignin peroxidases, manganese peroxidases, laccases, and other enzymes) traditionally associated with the lignin degrading system of this fungus are important. However, it is likely that other oxidative enzymes, especially cytochrome P-450 monooxygenases may also be important and they require further scrutiny for a better understanding of how azo dyes (and other organic pollutants) are degraded by these fungi. There is substantial interest in

harnessing the biodegradative abilities of white rot fungi to treat contaminated soil and water and considerable progress is being made in the development of bioreactors that are able to effect remediation of water contaminated with a variety of colored substances, including azo dyes.

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Fungal Degradation of Explosives

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1 INTRODUCTION

Explosive compounds are very significant environmental contaminants. Their production, testing, use in conflict, and disposal has led to contaminated soils, sediments, and water around the world (Spain et al. 2000). These materials are intrinsically toxic to microbes, plants, animals, and man. In the United States, it is now a requirement that sites contaminated by these compounds are risk assessed and remediated to acceptable standards determined by site-specific clean-up goals (Jerger and Woodhull 2000). Various remediation strategies have been adopted to date, and these have involved physical, chemical, and biological approaches (Newcombe and Crawford 2002). Physical techniques such as incineration, activated carbon absorption, and filtration are effective, but generate unwanted residues that still have to be treated. Chemical treatments have been tried, for example precipitation using surfactants and solvent extraction (Kaplan 1990) but again these approaches generate further residues that have to be disposed of. Biological treatments have included biostimulation of existing indigenous microflora and bioaugmentation where an explosive degrading microbial inoculum is added to the contaminated environment (Kaplan 1990). Biological approaches appear to be cost effective alternatives to other methods, but they have had limited success because of microbial sensitivity to toxic levels of explosive, toxic by-products of incomplete degradation and the length of time biological clean-up of a contaminated environment can take (Reddy 1995). This review seeks to summarize available information about the metabolic pathways involved in fungal biodegradation of explosives and to evaluate the distribution of these pathways across the taxonomic groups.

2 EXPLOSIVES AS SUBSTRATES FOR MICROBIAL DEGRADATION

2.1 2,4,6-Trinitrotoluene (TNT)

The nitro-aromatic compound 2,4,6-trinitrotoluene (TNT) is of greatest concern as an environmental contaminant (Figure 1a). It is intrinsically difficult for microorganisms to mineralize as the three symmetrically placed nitro- groups on the aromatic ring reduce its electron density and limits attack by electrophilic di-oxygenase enzymes (Nishino et al. 2000). Moreover, TNT is poorly soluble in water (140 mg/l at 25°C), relatively stable and persistent in the environment (Gorontzy et al. 1994). Its partial breakdown products are more hazardous, recalcitrant, carcinogenic, and mutagenic than the parent compound (Bennett 1994; Whong and Edwards 1984; Won et al. 1976).

Natural decomposition is often the result of co-metabolic bio-transformation but not mineralization and breakdown products include mutagenic reduced amines (Yinon 1990). Nitroaromatic compounds such as TNT are very rarely produced in nature. A few antibiotics containing nitro-aromatic groups are produced by *Pseudomonas* sp., for example nitropyrroluteorin (Ohmori et al. 1978) and oxypyrrolenitrin (Hattari et al. 1970). *Streptomyces venezuelae* has also been reported to produce chloramphenicol, and bryozoans have been reported to produce the antibiotic phidolophin (Tischler et al. 1986). Nitrophenolic compounds such as aristolochic acid can be produced by plants (Williams and Barnaby 1977) and this compound has been identified as a potent carcinogen, intercalating or forming adducts with DNA leading to oncogenesis (Arlt et al. 2000).

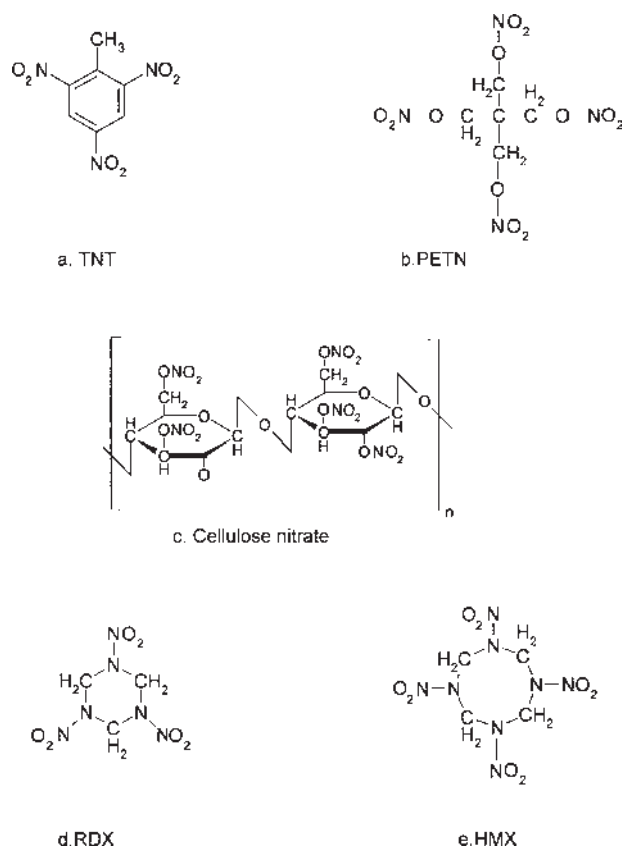


Figure 1 Chemical structures of the major classes of explosives. (a) TNT. (b) PETN. (c) Nitrocellulose. (d) RDX. (e) HMX.

2.2 Nitrate Esters and Methylene Nitramine Cyclic Esters

Nitrate esters, including glycerol trinitrate (nitroglycerin) and pentaerythritol tetra nitrate (PETN) (Figure 1b) are pharmacologically active at low concentrations and at high concentrations they are acutely toxic (Gorontzy et al. 1994). Other nitrate esters, like cellulose nitrate (Figure 1c), are nontoxic and relatively stable. Naturally occurring, biologically generated, organic nitrate esters appear very rarely. There is a single report of an insect sex pheromone that contains a nitrate ester (Hall et al. 1992).

Another group of energetic compounds that are significant environmental contaminants are cyclic trimers of methylene nitramine (Gorontzy et al. 1994) (Figure 1d and e). This group includes the most powerful military explosives in use today, RDX (cyclotrimethylenetrinitramine) and HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine). RDX is often mixed with TNT and is therefore a common environmental co-contaminant. It also has a limited aqueous solubility of 42.3 mg/l at 20°C (Gorontzy et al. 1994). HMX is used in shaped charges or as a rocket propellant and is a by-product of

RDX production with a higher melting point than RDX. This explosive has a very low aqueous solubility of 5 mg/l at 25°C. These compounds are truly xenobiotic, and do not occur naturally. Nitramines are acutely toxic and are class C carcinogens (Hawari et al. 2000a).

2.3 Origins and Evolution of Degradation Pathways

The xenobiotic nature of these compounds and their poor aqueous solubility may help explain why, to date, they have accumulated in the environment. However, within the last decade, after considerable exposure, bacteria are now being isolated from contaminated environments that are capable of mineralizing some of these explosives. For example, a TNT degrading *Pseudomonas* sp. has been isolated from contaminated water samples from a nitrobenzene production site (Parales 2000). Examination of the gene clusters associated with the nitroarene degradation pathway of this bacterium indicates that the evolution of the pathways is a recent event. This is suggested by the fact that the pathway is not optimized, there are vestigial genes, a lack of co-ordinated regulation, and long acclimatization times (Parales 2000).

3 BIODEGRADATION OF TNT BY FUNGI

In contrast to the newly evolved pathways we see in the bacteria, fungi have the capability to biodegrade explosives using existing, initially co-metabolic, pathways that lead to mineralization. Parrish (1977) published the results of a screen of the explosive degrading capability of 190 fungi. This study showed that fungi were capable of TNT degradation, but the sensitivity of the fungi to levels exceeding 20 ppm was such that Parrish discounted their use in bioremediation. Bennett (1994) comments that the interpretation of results from this work appears to have delayed more serious consideration of fungi as explosive degraders for some years. However, more recent screens have shown that the ability to degrade TNT to some degree was distributed across many genera within the Zygomycota, Ascomycota, and Basidiomycota (Scheibner et al. 1997b; Weber et al. 2002). Recent data suggests that under the right conditions fungi are capable of achieving mineralization of TNT at rates far higher than bacteria (Hawari et al. 2000a).

The best studied fungal TNT decomposer is the lignin decomposing Basidiomycete *Phanaerochaeta chrysosporium*. This fungus was first noted for its lignolytic capabilities when it was found to cause the overheating of woodchip piles (Burdall 1981) but to date its natural niche is unknown. It has been demonstrated in numerous studies to be capable of TNT degradation (Esteve-Nunez et al. 2001; Fernando et al. 1990). The initial reduction of TNT is independent of the ligninase enzymes associated with its mineralization, and is mediated by nonspecific nitroreductases that catalyze the conversion of highly oxidized

nitro-functional groups to mono- and di-amino toluenes (ADNT and DANT), and nitroso- and hydroxylamine containing intermediates (Esteve-Nunez et al. 2001). Stahl and Aust (1993) demonstrated that this reaction is dependent on the presence of a living mycelium and appears to be associated with a membrane bound redox system. This work was recently confirmed by Van Aken et al. (1999). However, Michels and Gottschalk (1995) have reported intracellular $\text{NADPH}^+ \text{H}^+$ dependent TNT reductase activity. A further mechanism was reported by Eiler et al. (1999). Using *Bjerkandera adusta*, they observed TNT breakdown was associated with a microsomal cytochrome P 450.

The initial co-metabolic reactions increase the electron density of the aromatic ring and facilitate electrophilic attack by lignin-degrading enzymes (Field et al. 1993). Lignin and TNT degradation occurs by a series of co-metabolic, synergistic reactions that involve three enzymes, manganese peroxide (MnP), lignin peroxidase (LiP), and laccase. The peroxidase enzymes are haem-containing glycoproteins that require hydrogen peroxide to function and they catalyze single electron oxidations that generate free radicals. Hydrogen peroxide is generated by oxidase enzymes including glyoxyl- and aryl-oxidase. Laccase is a copper containing phenol oxidase, which uses molecular oxygen as its terminal electron acceptor (Fritsche et al. 2000). Laccase can catalyze both polymerization and depolymerization reactions via oxidation (Harvey and Thurston 2001). Subsequent mineralization steps of TNT reduction products,

including nitroso-toluene (NST), ortho-hydroxyl amino 2,4-dinitrotoluene (HADNT), ADNT, and DANT are illustrated in a putative pathway constructed by Hawari et al. (2000a) (Figure 2) where the initial products are azo, azoxy, phenolic, and acylated derivatives. These compounds are already known to be mineralized by white rot fungi (Esteve-Nunez et al. 2001). However in *P. chrysosporium*, HADNT is known to inhibit veratryl alcohol oxidation by LiP (Bumpus and Tartako 1994). The veratryl alcohol to veratryl aldehyde conversion is essential for the production of free radicals that are involved in the oxidation of primary substrates of LiP. Thus biodegradation of TNT will be inhibited if levels of HADNT are allowed to accumulate. Bumpus and Tatarko (1994) reported that levels as low as $30 \mu\text{M}$ will inhibit enzyme activity. This sensitivity is regarded as a key limitation to the use of this fungus in biodegradation in the field (Michels and Gottschalk 1995).

For several years, research concentrated on *P. chrysosporium* as a prime candidate for explosive degradation. However, other fungi have been screened and Table 1 summarizes published work to date. In some of the other fungal species screened, tolerance to TNT and its breakdown products can be much higher than that of *P. chrysosporium*. For example, *Rhizopus nigricans* was reported as being able to remove TNT from a medium containing 100 mg/ml TNT (Klausmeier et al. 1974) and *Irpex lacteus* tolerated up to 50 mg/ml of TNT, and was able to degrade TNT by more than one route, forming transient hydride-Meisenheimer complexes (Kim and Song 2000).

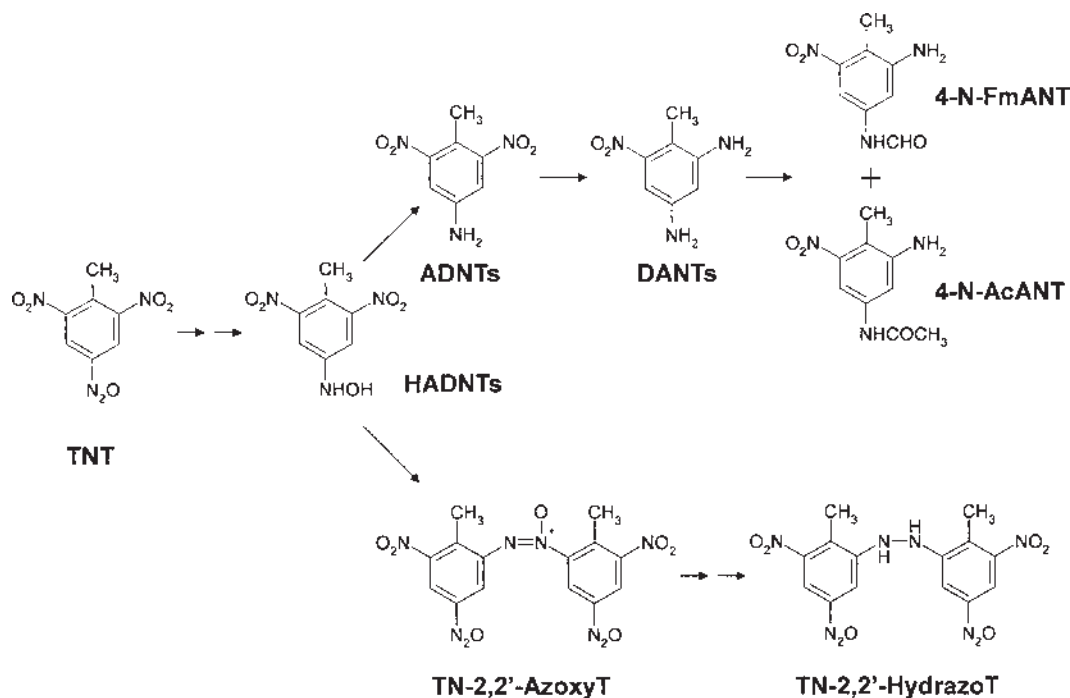


Figure 2 One of the putative pathways of TNT degradation by fungi.

Table 1 Species of fungi reported as capable of TNT degradation

Division	Family	Genus/species	Type	Habitat	Comments
Basidiomycetes/ Polyporales	Gloeophyllaceae	<i>G. seiparium</i>	Brown rot	Wood	Hess et al. 1998
	Phanerochaetaceae	<i>P. chrysosporium</i>	White rot	Wood	ATCC 24725
	Meruliaceae	<i>P. sordida</i>	White rot	Wood	HHB 8922, Donnelly et al. (1997)
		<i>P. radiata</i>	White rot	Wood	ATCC 64658, Van Aken et al. (1999)
		<i>P. brevispora</i>	White rot	Wood	HHB7030, Donnelly et al. (1997)
	Polyporaceae	<i>P. ochracheofulva</i>	White rot	Wood	Scheibner et al. (1997a, b)
		<i>T. versicolor</i>	White rot	Wood	DSM 11269, Scheibner et al. (1997a, b)
		<i>T. suevolens</i>	White rot	Wood	MWT 03-2, Scheibner et al. (1997a, b)
		<i>L. sulphureas</i>	White rot	Wood	Scheibner et al. (1997a, b)
		<i>F. fomentarius</i>	White rot	Wood	MWF 01-4, Scheibner et al. (1997a, b)
		<i>S. commune</i>	White rot	Wood	Kim and Song (2000)
		<i>C. versicolour</i>	White rot	Wood	KR11W, KR 65W, Kim and Song (2000)
		<i>P. coccineus</i>	White rot	Wood	Kim and Song (2000)
		<i>L. lacteus</i>	White rot	Wood	Kim and Song (2000)
Basidiomycete/ Agaricales	Atheliaceae	<i>T. fibrillosa</i>		Ectomycorrhizal	Meharg et al. 1997
	Hapalopilaceae	<i>B. adusta</i>	White rot	Wood	Field et al. 1993
	Bondarziaceae	<i>H. annosum</i>	White rot	Wood	TM5P2, Scheibner et al. (1997a, b)
	Strophariaceae	<i>H. fasciculare</i>	White rot	Wood	TM 52, Scheibner et al. (1997a, b)
		<i>K. mutabilis</i>	White rot	Wood	TME, TM 1/68, Scheibner et al. (1997a, b)
		<i>N. frowardii</i>	White rot	Wood	ATCC 201144, DSM 1239, Scheibner et al. (1997a, b)
		<i>S. ruguloso-annulata</i>		Litter	DSM 11373, TME, Scheibner et al. (1997a, b)
	Tricholomataceae	<i>C. dusenii</i>	White rot	Wood	DSM 11238, TMb 12, Scheibner et al. (1997a, b)
	Nidulariaceae	<i>C. stercorus</i>	White rot	Wood	36910 Donnelly et al. (1997)
	Tricholomataceae	<i>C. (Lepista) nebularis</i>		Litter	TM, Scheibner et al. (1997a, b)
		<i>C. odora</i>		Litter	TM3, Scheibner et al. (1997a, b)
	Plutaceae	<i>A. muscaria</i>		Ectomycorrhizal	Scheibner et al. (1997a, b)
	Agaricaceae	<i>H. fasciculare</i>	White rot	Wood	TM5.2
		<i>A. aestivalis</i>		Litter	TMAEST1, Scheibner et al. (1997a, b)
		<i>A. bisporus</i>		Litter	MWA 80-7, Scheibner et al. (1997a, b)
Basidiomycetes/ Boletales	Suillaceae	<i>A. praecox</i>		Litter	TM 70 84, TM70.3.1 Scheibner et al. (1997a, b)
		<i>S. granulatus</i>		Ectomycorrhizal	Scheibner et al. (1997a, b)
		<i>S. variegates</i>		Ectomycorrhizal	Meharg et al. 1997
	Sclerodermataceae	<i>P. tinctorus</i>		Ectomycorrhizal	Meharg et al. 1997
		<i>P. frequentens</i>		Ubiquitous	ATCC 96048, Scheibner et al. (1997a, b)
	Moniliales	<i>Penicillium</i> sp.		Ubiquitous	DSM 11168, Scheibner et al. (1997a, b)
		<i>P. chrysogenum</i>		Ubiquitous	IFO 31249, Kim and Song 2000
		<i>A. terreus</i>		Ubiquitous	MW 458, Scheibner et al. (1997a, b)
Ascomycete anamorphs					

Zygomycetes	<i>F. solani</i>	Ubiquitous	Scheibner et al. (1997a, b)
	<i>Fusarium</i> sp.	Ubiquitous	TM 821, Scheibner et al. (1997a, b)
	<i>Alternaria</i> sp.	Ubiquitous	TMR 2, Scheibner et al. (1997a, b)
	<i>N. crassa</i>	Ubiquitous	TM, Scheibner et al. (1997a, b)
	<i>A. resinæ^a</i>	Ubiquitous	Klausmeier et al. 1974
	<i>C. echinulata</i>	Ubiquitous	<i>v. elegans</i> DSM (1980), Scheibner et al. (1997a, b)
	<i>M. mucedo</i>	Ubiquitous	DSM 810, Scheibner et al. (1997a, b)
	<i>R. nigricans</i>	Ubiquitous	

TM = Culture collection of the Institute of Microbiology, Jena, Germany; MW = Stock culture collection, Weimar, Germany; DSM = German Culture Collection, Braunschweig, Germany; ATCC = American Type Culture Collection, <http://www.atcc.org>

^a Also known as *Cladosporium resinæ*.

4 BIODEGRADATION OF NITRATE ESTERS BY FUNGI

Substituted nitrate esters have a fairly low aqueous solubility. Glycerol tri-nitrate saturates at 1.5 g/l, whilst fully substituted nitro-cellulose is completely insoluble in water (Williams and Bruce 2000). Although these compounds are relatively stable, there is some evidence that in the natural environment they can be reductively transformed, forming alcohols and nitrates (Williams and Bruce 2000). There is a dearth of published literature on fungal biodegradation of nitrate esters and the possible pathways by which such degradation could occur. The few fungi reported as having some degradative effects on nitrate esters are distributed across the mitosporic fungi and wood rotting Basidiomycete genera. *Geotrichum candidum* was shown to have denitration capability, generating glycerol dinitrate and glycerol mono-nitrate with glycerol-2 mono-nitrate the predominant product (Ducroq et al. 1990). This work demonstrated a regio-specificity in the de-nitration, and they suggested that different enzymes were involved in the de-nitration reactions. *P. chrysogenum* appears also to have the ability to denitrate nitrate esters and in this Basidiomycete, the enzymes responsible for these reactions appear to be glutathione-S-transferases (GST) (Servent et al. 1991; 1992). Glutathione-S-transferase enzyme activity has been located in the cytosol. Furthermore, these workers also identified a cytosolic and microsomal P450-like enzyme with similar activity. There are therefore at least two different classes of enzymes involved in the de-nitration of GTN, an oxygen insensitive glutathione-dependent enzyme that liberates nitrate, and an oxygen-sensitive NADP^{red} dependent P450-like activity that liberates nitrous oxide. In all cases of fungal degradation of nitrate esters, additional carbon sources have had to be supplied, and degradation is only partial. Even when a cellulolytic species is combined in co-culture with a de-nitrating species (*Sclerotium rolfsii* plus *Fusarium solani*) decomposition is incomplete (Sharma et al. 1995; Sundaram et al. 1995).

5 BIODEGRADATION OF RDX/HMX BY FUNGI

In contrast to TNT, RDX and HMX are intrinsically less recalcitrant. Once there is a change in the ring structure, for example by cleavage of a N–NO₂ bond or C–C bond, the ring structure undergoes spontaneous decomposition, producing small nitrate and carbon containing compounds that are easily mineralized by many species of microorganisms (Hawari 2000). However, there is only a limited literature on biodegradation of cyclic triamines. Bayman et al. (1995); Bayman and Redkar (1997) report that mycelium of *P. chrysogenum*, *Cladosporium resinæ*, *Cyathus pallides*, and *Cunninghamella echinulata* var *elegans* were not inhibited by 50–100 µg/ml RDX, and that the RDX “disappeared” when incubated with these fungal species.

However, it was not possible to trace the breakdown products or to determine if the disappearance was due to mineralization reactions or bio-transformations. Fernando and Aust (1991) reported that *P. chrysogenum* could mineralize RDX, and they provided some evidence that a free-radical chain mechanism was involved in the degradation, but again were unable to identify the initial products of decomposition.

6 USE OF FUNGI IN BIOREMEDIATION IN THE FIELD

A number of different strategies have been adopted where attempts have been made to exploit fungi in bioremediation. Early findings demonstrated that attempts to establish wood rotting species in soil without amendments or soil sterilization failed. Like the early failures in establishing biological control agents in soil (Faull 1986), the indigenous micro flora out-competed the inoculants unless there were large additions of substrates such as wood chips and other ligno-cellulosic materials to the soil (Bennett et al. 2002; Cerniglia and Sutherland 2002; Radtke et al. 1994). Currently, a number of approaches are being tried, including soil piles and windrows (where "composting" may occur), soil farming, soil slurry reactors, and fixed film reactors (Rogers and Bunce 2001).

6.1 Soil Piles and Windrows

Soil piles and windrows are created by mixing soil with wood chips, corn cobs, or other ligno-cellulosic materials and adding fungal inoculum on a lignocellulosic base. The pile is then left for an extended period of time with regular turning and wetting for composting, or no turning for the static pile. This approach can lead to a rapid disappearance of explosive contaminants. A number of different fungal inoculants have been tried. For example, Jerger and Woodull (2000) used *Trametes versicolour* and *P. chrysosporium* as soil pile inoculants, Fritsche et al. (2000) used *Stropharia rugosoannulata* and Spreinart et al. (1998) used *B. adusta*. This approach is being widely used in the United States for the clean up of military sites (USAEC 1999). However, criticisms of this technique include the long incubation times needed for complete disappearance of the target substrate, and the high costs of set-up and maintenance. The process is further criticised for being based on unknown biological processes that may produce toxic breakdown products that bind to soil organic matter and undergo no further mineralization (Hawari 2000). Furthermore, in many cases it has been shown that added microbial inoculants did not persist in the environment and it was the indigenous micro flora that achieved much of the degradation (Gerth et al. 2001).

6.2 Landfarming and Soil Slurry Reactors

Land farming, where inoculants and moisture are ploughed into soil on a large scale, has had little success using fungal inoculants and soil slurry reactors have also proven to work better with bacterial inoculants than fungal (Hawari et al. 2000b). However, Fritsche et al. (2000) remain optimistic about the potential of the land farming approach using fungal inoculants.

6.3 Bioreactors

Fungal inoculants have also been used in aqueous-based systems using fixed fungal films. They may be attached to rotating discs in a bioreactor (Sublette et al. 1992) (*P. chrysosporium* to degrade TNT and RDX) or in continuous culture air lift fermenters (Rho et al. 2001) (*P. chrysosporium* with TNT).

7 FUTURE PROGRESS

It is possible to identify some key features that limit successful use of fungi in bioremediation processes and identify possible strategies that might be employed to overcome them.

7.1 Use of Photocatalysis to Reduce Toxic Effects of Breakdown Products

There is a sensitivity of key fungal enzymes to explosive degradation products that currently limits the use of fungi in bioremediation of contaminated materials. This is exemplified by the sensitivity of veratryl alcohol oxidase activity of *P. chrysosporium* LiP to HADNT. A solution to this problem may be in the use of combined photo-catalytic and biological processes. The photo catalytic process degrades the toxic intermediates of breakdown and allows the fungal enzymes to continue mineralizing the products of photo-catalytic breakdown (Hess et al. 1998).

7.2 Use of Anti-Oxidants to Control Quenching

There is evidence that one of the key elements of the degradation process, the production of free radicals via the activity of LiP and MnP, is quenched by the presence of humic compounds (Hawari 2000). The use of fungi in controlled environments (particularly in fixed film reactors), where reduced glutathione and other thiols can be added to reverse the quenching effects, has produced promising results (Scheibner and Hofrichter 1998; Fritsche et al. 2000).

7.3 Screening for New Fungal Species Strains

Strain selection for effective explosive-degrading fungi is far from complete. The rather random screening approach used to date has shown that the ability to degrade substrates like TNT is widespread across fungal genera (Scheibner et al. 1997a,b). Most of the species studied have been from temperate regions. However, the tropical species may well have greater lignolytic capabilities as they are able to decompose far more woody litter per year than the temperate species can (Swift et al. 1976). Furthermore, many species of fungi with no lignin-degrading capability seem able to partially biodegrade TNT and other explosives by unknown metabolic routes. Understanding of the enzymology associated with these reactions would seem to be a priority. This would lead to a more rational screening approach.

7.4 Matching Colonization Strategies with Bioremediation Requirements

It is essential to understand the way in which different fungi colonize and function in different niches. The initial phase of substrate colonization has little to do with ligninases (Evans and Hedger 2001). Lignin has such a low calorific value that it cannot act as a sole carbon source, therefore white rot fungi only degrade lignin when they cannot utilize cellulose. For example *T. versicolour* enters wood through cuts in vessels or tracheids and it colonizes rapidly through these structures, utilizing soluble materials whilst sequestering and retaining substrate. Only after colonization and substrate possession is complete does cellulose availability become limited, and as nitrogen levels reduce to below 200:1 C:N lignin degradation begins. Other members of the Aphylllophorales, including *Ganoderma* sp., *Fomes* sp., and *Inonotus* sp., are slow to colonize and the onset of lignin degradation is even slower, but very recalcitrant molecules can be degraded (Evans and

Hedger 2001). Species from the *Sphaerales* are slow lignin degraders with great tolerance to water stress. It is essential to understand such ecological effects and to match these biodegradative abilities with the desired end result before selecting a species of fungus to use in bioremediation of a particular substrate.

7.5 Use of Sorption Strategies

Bioattenuation/humification strategies are currently being seriously considered as alternatives to mineralization (Hawari et al. 2000a,b; Isbister et al. 1984; Weber et al. 2002). In many experiments, mass balance equations prove that mineralization does not occur, but TNT breakdown products “disappear.” The process appears to be due to irreversible binding (sorption) of TNT breakdown products with the soil humic and clay fraction (Achnich et al. 1999; Elovitz and Weber 1999; Weber et al. 2002). Sorption mechanisms include electrostatic interactions and co-valent bonding (Head 1998) (Figure 3). Sorption interactions will also depend on the chemistry of the pollutant, and the amount of clay and organic content of the soil. Binding can also be mediated via oxido-reductive enzymes including laccase (Bollag et al. 1992). The driving force behind accepting that “disappearance” is a satisfactory end point in bioremediation is the poor results so far obtained from in situ remediation of soil using fungal inoculants. Such an approach needs careful evaluation as Palmer et al. (1997) found that apparently irreversibly bound products could release sufficient residues under certain conditions to be detectable in mammalian bioassays. The presence of these pollutants was not detectable by conventional analytical chemical techniques. Sorption is influenced by pH and high will release sorbed molecules (Head 1998). Natural surfactants produced by indigenous soil microbes can also release sorbed products. There is an increasing awareness that irreversible binding may not

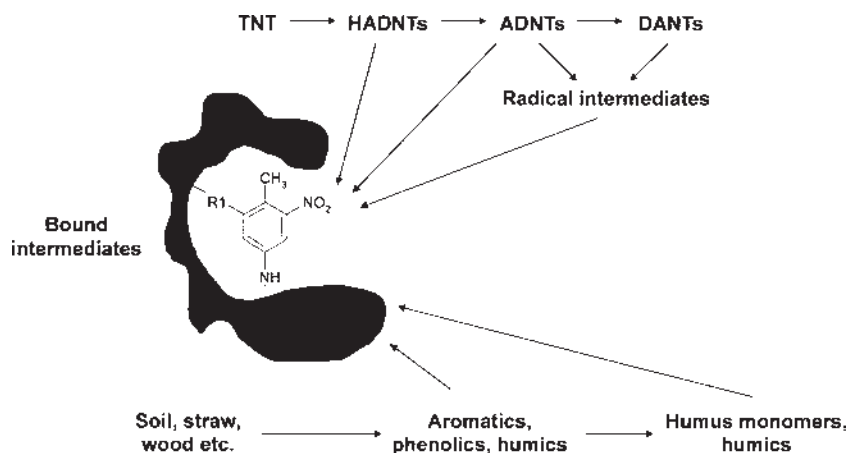


Figure 3 Interaction of TNT degradation products with materials in soil.

actually occur, and there are many on-going studies on the use of toxicity assays to monitor composts produced by bioremediation (Gundersen et al. 1997; Rocheleau et al. 1999).

8 CONCLUSIONS

There is clearly a pressing need for new approaches to bioremediation of explosives-contaminated soils and sediments. There is increasing evidence that co-metabolically produced products of the incomplete degradation of explosive compounds such as TNT are not as tightly bound to clays and organic matter as was first thought. Their presence in an ecosystem can be detected by bioassay even though, in terms of chemical analysis, they have disappeared. Current techniques have failed to fully exploit the capacity of fungi to mineralize these compounds via naturally occurring pathways. This appears to be because a limited selection of strains and species of fungi have been screened for their explosive degrading capability. We have an incomplete understanding of metabolic pathways involved in the strains that have been selected. We do not fully understand the microbial ecology of these fungi when used in bioremediation. Further work is needed within all three of these areas before large scale, field-based trials are undertaken that may create as many challenges as they overcome.

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Restoration of Mycorrhizae in Disturbed Arid Ecosystems

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1 INTRODUCTION

Mycorrhizae are mutualistic symbioses between plants and fungi. As such, mycorrhizal fungi play an important role in structuring plant communities by improving the performance of individual plants. Improved growth and survival of host plants is mostly attributed to the ability of fungal partners to improve plant nutrition and rooting structure (Allen and Allen 1986; Allen et al. 1989; Lewis 1973) and enhance resistance to environmental stresses and soil pathogens (Sylvia and Williams 1992). However, a variety of mycorrhizal types differentially affects their host plants. If the appropriate group of fungi is unavailable to form the type of mycorrhizal association to which a plant belongs, that group of plants may not remain or recover from a disturbance (Allen et al. 1993). Disturbance alters fungal communities in different ways and fungal recovery typically occurs after initial plant establishment. As a consequence, the formation of functional mycorrhizae may require decades or may never occur.

The importance of mycorrhizae to the restoration of arid plant communities has received considerable attention, most notably because successful establishment of mycorrhizae has implications to rates and patterns of plant succession (Allen and Allen 1990). It is critical, therefore, to understand the key factors that regulate the natural reestablishment of mycorrhizae (Allen et al. 1992), and determine whether mycorrhizal inoculation is a viable alternative to managing existing fungal populations or manipulating their natural reinvasion. Although the specific factors that regulate natural reestablishment of mycorrhizae are not entirely known, they involve survival of residual fungal propagules, dispersal of propagules from adjacent undisturbed areas or from local sites of residual survival, and available microsites where plants and fungi

can survive and contact each other (Allen et al. 1992). Alternatively, inoculations of a disturbed site with native or exotic fungi facilitate formation of mycorrhizae in a shorter period, given certain circumstances. The simple application of inoculum, however, does not guarantee the formation of a functional mycorrhiza. The potential benefits of inoculation could be outweighed by cost and efficiency limitations, if the probability of plants benefiting from mycorrhizae is low (Findlay and Kendle 2001). Understanding mycorrhizal function and limits of mycorrhizal function are critical in studying ecosystem response to disturbance and recovery from disturbance (Allen et al. 1999).

In addition to examining the integral role of mycorrhizal fungi in ecosystem restoration, there has been considerable interest in determining the influence of mycorrhizal fungi on revegetation of severely polluted soils. The use of mycorrhizal fungi in bioremediation of metal-polluted soils has received increasing attention. Recent studies suggest that mycorrhizal fungi may exhibit some degree of heavy metal tolerance and as a result, confer heavy-metal tolerance in host plants. However, under natural conditions, the extent to which mycorrhizal associations benefit plants, in terms of alleviating metal toxicity, remains largely uncertain (Leyval et al. 1997). In this chapter, we will address in more detail the response of mycorrhizal fungi to disturbance and the factors that inhibit or facilitate their natural recovery. We will also discuss management options for enhancing native fungal reestablishment and the potential benefits and drawbacks of using mycorrhizal inoculum. Lastly, we will briefly discuss the potential role of mycorrhizal fungi as bioindicators and their application to bioremediation.

2 TYPES OF MYCORRHIZAE

The term mycorrhiza describes the symbiotic relationship between diverse assemblages of fungi and plant roots. Mycorrhizal associations are considered mutualistic because they are both the normal state (Smith and Read 1997) and a constant feature (Smith 1995) of most plants under most ecological conditions. There are several types of mycorrhizae characterized with respect to the organisms involved in the symbiosis and their anatomy and function. The most common is called an arbuscular mycorrhiza (AM). The AM fungi (Glomales, Zygomycetes) occur in most terrestrial environments (Allen et al. 1995; Janos 1980) and are the dominant association in grassland, shrubland, and agricultural ecosystems. These fungi are characterized by the formation of arbuscules, thinly branched hyphal structures within root cortical cells responsible for transfer of immobile nutrients such as phosphorous (P) from fungi to plant. Arbuscular mycorrhizae fungi are obligate biotrophs, obtaining all of their carbon from a host plant, which makes culturing and mass-producing fungal isolates for large-scale inoculation efforts extremely difficult. The second most common mycorrhizal type is ectomycorrhiza (EM). The majority of EM fungi are basidiomycetes and ascomycetes, with few species of zygomycetus fungi in the genus *Endogone* (Smith and Read 1997). These fungi associate almost exclusively with woody plants in the Pinaceae and Fagaceae (Allen et al. 1995). Ectomycorrhizae fungi do not penetrate the plant cell walls but surround them forming a Hartig net and encase individual roots with a mantle of fungal tissue. These fungi are used extensively in forest reclamation worldwide, the most common being *Pisolithus tinctorius* (Marx and Cordell 1989; Marx et al. 1976), *Rhizopogon* spp. (Trappe 1977), and *Suillus* spp. (Chapela et al. 2001). Lastly, other less dominant mycorrhizal types that form important species-specific associations with ericaceous and orchidaceous plants should also be included in their restoration efforts.

3 MYCORRHIZAL DIVERSITY

3.1 Fungal and Plant Species Richness

While plants vary in their dependence on mycorrhizal fungi, the fungi are generally obligate mutualists (Lewis 1973). Interestingly, patterns of mycorrhizal diversity do not always follow those of plant diversity (Allen et al. 1995). There are approximately 150 species of AM fungi (Morton et al. 1995; Schenck and Perez 1990) forming associations with about 70% of plants worldwide. Virtually any AM fungus can associate with any vascular plant capable of forming an AM (Allen et al. 1995). Despite low fungal species richness, many AM communities can exhibit high plant species diversity. In Wyoming sagebrush-steppe, 11 species of AM fungi were found where plant species richness exceeded 150 species (Allen et al. 1993). In a survey of 80 sites across the Great Basin in the western United States, Allen et al. (1993) found

only 48 species of AM fungi, with no one site containing more than 12 species. In a seasonal tropical forest, Allen et al. (1998) found approximately 25–30 species of AM fungi in a forest with plant species richness exceeding 1,000.

Unlike AM fungi, the formation of EM involves a greater diversity of fungal species (>5400 species), exhibiting varying degrees of host specificity (Molina et al. 1992). For example, the EM fungal genera, *Hydnangium*, is found only on *Eucalyptus* and *Suillus* and *Rhizopogon* are restricted to Pinaceae, while *Amanita* and *Laccaria* associate with most EM hosts (Molina et al. 1992). Further, EM fungal diversity can be high in areas where plant community diversity is low. Early studies by Trappe (1977) estimated 2000 species of EM associated with Douglas Fir alone. In the Jarrah forest of southwestern Australia dominated by *Eucalyptus marginate* and *E. calophylla*, 90 species of EM fungi were found (Hilton et al. 1989). Over 50 species of EM fungi were identified in a *Quercus agrifolia* stand near Temecula in southern California. These included truffle fungi in the genera *Hydnotrypeis*, *Hydnotrya*, and *Tuber*, as well as epigeous mushrooms such as *Amanita*, *Boletus*, *Cortinarius*, *Laccaria*, and *Russula*. Still, most studies underestimate EM diversity because many of these fungi fruit irregularly, and fungi with abundant sporocarps may not form functional EM (Gardes and Bruns 1996; Gehring et al. 1998).

4 MYCORRHIZAL FUNCTION

4.1 Nutrient Uptake

Despite the differences in associations among mycorrhizal types, the function of all mycorrhizal systems depends on the ability of the fungal mycelium to absorb available inorganic or organic nutrients from the soil and translocate them to the host roots (Smith and Read 1997). Hyphae outside the root may form an extensive network extending several centimeters from the root surface (Friesse and Allen 1991), enabling plants to obtain resources well beyond depletion zones (Nye and Tinker 1977) and to forage effectively for patchily distributed resources (Cui and Caldwell 1996). Fine, highly branched hyphae increase the absorptive surface area of the root (Harley and Smith 1983; Rousseau et al. 1994), produce enzymes for mineralization of nutrient sources that are normally unavailable to plants (Abuzinadah and Read 1986; Bauer et al. 2000), and because of their extremely fine diameter they are considerably less expensive than roots. As a result, mycorrhizal plants undergo physiological changes which include increased rates of growth and seed production, increased nutrient status, enhanced water uptake and drought tolerance, and improved resistance to plant pathogens.

4.2 Carbon Allocation

Arbuscular mycorrhiza fungi are obligate symbionts and must obtain carbon (C) from the host plant, whereas EM fungi

primarily uses host plant C. Yet, some taxa are capable of supplementing host C with supplies from the soil (Durall et al. 1994; Smith and Read 1997). Studies have estimated that 10–85% of net photosynthate is allocated to mycorrhizal fungi depending on the ecosystem (Allen 1991). Mycorrhizal plants consistently transfer more C belowground than nonmycorrhizal plants. Under low light or high nutrient conditions, mycorrhizae may suppress plant growth and the fungal partner has sometimes been considered a drain on the host (Johnson et al. 1997). However, a number of studies have shown that photosynthetic rates are enhanced in mycorrhizal plants to compensate for the cost of mycorrhiza development, regardless of nutrient availability (Allen 1991).

Mycorrhizal fungi are an important link in the chain of C transfer from plant to soil. We now realize the important role of mycorrhizae in sequestration of C in soil (Bonkowski et al. 2000; Rillig et al. 2001; Treseder and Allen 2000) and the potential for mycorrhizae to influence C cycling rates. Estimates of AM fungal biomass can account for 1–17% of the root weight (Fujiiyoshi et al. 2000) and EM can comprise 20–30% of the root volume (Harley and McCready 1952). Processes involved in the cycling of fungal C include production, survivorship, and decomposition rates of fungal tissue (Treseder and Allen 2000). The production and turnover of fine hyphal networks exceeds the turnover rates for both root and shoot material (Fitter et al. 2000). However, a substantial amount of the C allocated to mycorrhizal tissue could remain in the soil for a longer period. Chitin, a recalcitrant polysaccharide, can constitute 60% of fungal cell walls and may persist years to decades in the soil. The functioning hyphal mycelium also secretes an insoluble glycoprotein called glomalin (Wright and Upadhyaya 1996) that sloughs from hyphae during the life span of root colonization (Wright and Upadhyaya 1999; Wright et al. 1996). Glomalin is important in forming water-stable aggregates and in soil fertility; measurement of this protein concentration in soils will help with the comparison of soils of different compositions and/or tillage or disruption practices (Wright and Upadhyaya 1996). Because hyphal productivity varies among AM fungi, glomalin production also varies (17 $\mu\text{g}/\text{mg}$ hyphae–63 $\mu\text{g}/\text{mg}$ hyphae) (Wright et al. 1996). Glomalin levels can be present in soil as high as 1.5% of the soil dry weight (Wright and Upadhyaya 1996) and constitute 30–60% of the recalcitrant carbon in undisturbed soils (Treseder and Allen 2000). Carbon dating of glomalin indicates that turnover occurs at time scales of several years to decades, much longer than the turnover estimates for AM hyphae (Rillig et al. 2001).

4.3 Soil Stabilization

In addition to direct benefits of plant growth and health, the contribution of fungal mycelium to soil aggregation and soil organic matter development may be a critical component to most restoration programs (Miller and Jastrow 1991). The role of fungi in enhancing soil stabilization is two-fold. First,

the extraradical hyphae of AM fungi, together with fine roots, physically bind soil particles into larger macroaggregate units (Miller and Jastrow 1990). Second, AM fungi channel a significant amount of carbon into the soil by which soil particles and organic material become bound, contributing to soil aggregation and stability (Rillig et al. 1999). Typically, there are between 1–20 m of AM hyphae/g of soil (Sylvia 1990). In cold deserts, Allen and MacMahon (1985) reported hyphal length of 2–6 m/g of soil. Treseder and Allen (2001) reported similar values (2–4 m of live AM hyphae/g of soil) in a chaparral ecosystem. In contrast, Miller et al. (1995) found that soils associated with mycotrophic plants in tallgrass prairie had 111 m/cm³ and Allen and Allen (1988) reported that in Wyoming sagebrush steppe, AM fungi produced 28–54 m total hyphae/g of soil. The significant contribution of fungal hyphae to the formation of stable soil aggregates that prevent wind and water erosion which may be crucial to restoration success (Miller and Jastrow 1991) and carbon sequestration (Jastrow et al. 1996).

5 MYCORRHIZAL RESPONSE TO DISTURBANCE

The fundamental role of mycorrhizal fungal hyphae is to provide a direct physical link between the plant community and surrounding soil resources (Miller and Jastrow 1992). Disturbance to soil systems always alter in some way the spatial heterogeneity between soil fungi and plants, organic matter, and nutrient pools (Allen and MacMahon 1985; Allen et al. 1999). Major disturbances usually reduce mycorrhizal inoculum densities. Disturbance to hyphae can occur through compaction of soil pores or physical destruction of the hyphae (Allen and MacMahon 1985) and through vegetation removal or loss of plant cover. The impact of disturbance on mycorrhizal infectivity has been the topic of numerous studies (Jasper et al. 1989; Jasper et al. 1991; McGonigle et al. 1990).

6 RECOVERY OF MYCORRHIZAL FUNGI

The reestablishment of mycorrhizal fungi following disturbance has been extensively studied (Allen et al. 1999). Factors that are most influential to the recovery of mycorrhizae include the dispersal of plant and fungal propagules, the physicochemical soil environment, and the survival of fungal residuals. Plant and fungal propagules typically disperse independently of one another and then must encounter each other in a suitable microsite favorable to their survival. Plant and fungal propagules arriving at that microsite must also be viable and germinable (Cooke and Whipps 1993). Soil fertility, especially artificially high levels of available nitrogen (N) and P, could inhibit formation of mycorrhizae and slow the recovery of fungal propagules densities in soil. Therefore, survival of infective residual fungal propagules would greatly enhance mycorrhizal reestablishment (Allen and MacMahon 1985; Allen et al. 1992).

7 MANAGING EXISTING INOCULUM

7.1 Protecting Topsoil

If the disturbance event can be anticipated, as in the case of mining or logging, it may be possible to minimize the adverse effects to mycorrhizal communities through topsoil protection. In work undertaken by Miller et al. (1998), EM fungi persisted on dying roots following fires for over a year after the trees were dead: new seedling roots could tap this resource if it remains intact. Since most mycorrhizal fungi are present in the upper soil profile, topsoil serves as a reservoir of fungal inoculum, as well as a hospitable environment for invading propagules. Therefore, the careful management of topsoil should alleviate the need for intense inoculation efforts after disturbance. For example, when possible, disturbance to soil should be minimized by limiting or eliminating activities that exacerbate the level of impact (Allen et al. 1999). This allows mycorrhizae associated with plants and organic matter to stabilize soil and reduce erosion. It also protects intact hyphal networks that can rapidly form mycorrhizae on uncolonized roots of neighboring plants (Francis et al. 1986; Smith and Read 1997). In situations where land is to be eventually disturbed, the most effective means to preserve mycorrhizae may include salvaging the topsoil before disturbance and respreading it afterwards or transferring it away from the site to another disturbed area (Allen et al. 1999). Top-soiling can be a significant source of microbes, plant propagules, and organic matter (Allen and Allen 1980); however, critical to the benefits of top-soiling is the length of time, the soil spends without plant cover necessary to sustain symbiotic relationships. Soil may be stockpiled for several years, during which time organic matter (Schwenke et al. 2000), chemical composition (Kundu and Ghose 1997), and infectivity of mycorrhizal propagules can change greatly (Miller et al. 1985). Even after soil reapplication, slow or poor seedling recruitment may further hinder mycorrhizal establishment. Planting a dense cover crop of the appropriate plants on soil stockpiles could enhance the survival and subsequent establishment of mycorrhizal fungi on restoration sites.

7.2 Soil Amendments

Improving the structure of soil organic matter is an important management option for enhancing mycorrhizal development (Allen et al. 1999). Compost, bark, or some other recalcitrant carbon source provides a slow release of nutrients while increasing soil moisture, facilitating infiltration, and reducing soil compaction. These conditions are not only beneficial to mycorrhizal fungi, but also many other microorganisms that improve nutrient availability and plant performance. For EM particularly, the absence of organic matter following severe disturbance limits the establishment of mycorrhizae despite adequate dispersal onto the site (Allen et al. 1992; Read 1984). The use of soil amendments could therefore alleviate the need for further mycorrhizal inoculation.

7.3 Maintaining Source Areas

The ability of mycorrhizal propagules to colonize disturbed sites from across relatively long distances supports the need to maintain source areas with diverse and abundant mycorrhizal populations. The natural recovery of mycorrhizae was first presumed to be a limited process, relying heavily on the slow recolonization of plants and animals from disturbance edges. However, Allen et al. (1989); Warner et al. (1987) have demonstrated that both wind and animals can disperse AM spores quite rapidly and as far as 2 km across disturbed arid landscapes. On Mount St. Helens, wind rapidly dispersed EM inoculum, but animals were necessary for dispersal of AM (Allen et al. 1992).

7.4 Managing Natural Reinvasion

Of the diverse array of AM and EM fungi that could potentially inhabit a site, relatively few are used to replace the hundreds that are lost with disturbance. Managing a site to facilitate natural reinvasion may therefore be critical to the restoration process. In arid habitats, mycorrhizal propagules are dispersed across sites by both wind and animals (Warner et al. 1987). Evaluating and manipulating these two factors could potentially enhance the rate of mycorrhizal recovery. For example, Allen et al. (1989) found that wind dispersal and deposition patterns of fungal spores were predictable given an understanding of the physical and biological characteristics of a site. Knowing potential source and sink areas, it is possible to enhance the trapping of wind-blown propagules using artificial barriers such as snow fences or by manipulating the distances between individual plants thereby creating islands of fungal inoculum (Allen et al. 1997).

Animal activity on disturbed sites is also important to mycorrhizal recovery. Animals disperse fungal propagules, either by directly feeding on them (Allen 1988; Allen et al. 1997; Blaschke and Bäumler 1989; Rabatin and Skinner 1985) or by moving soil and root material containing propagules (Allen and MacMahon 1988; Friese and Allen 1993). Numerous animals consume the hypogeous fruiting bodies (truffles) of some EM fungi and deposit these in new locations, a critical element to the reforestation of many habitats (Allen et al. 1997). Pocket gopher (*Thomomys talpoides*) and harvester ant (*Pogonomymex occidentalis*) move substantial amounts of soil, bringing spores, root fragments, and plant propagules to the surface (Allen et al. 1984). Their mounds provide refuge and favorable microsite conditions for the establishment of late-seral obligately mycorrhizal plant species (Allen 1987). Interestingly, the dispersal of mycorrhizal propagules via animal activity could play an influential role in determining rates and patterns of plant reestablishment success.

7.5 Managing Plants to Enhance Recovery

The establishment of mycorrhizal associations depends largely on two facets: the characteristics of the host plant and the spatial pattern of planting. Plants exhibit different degrees of mycotrophy, primarily based on their capacity for nutrient uptake and their growth response to fungal colonization. In early work by Stahl (1900), plants were divided into nonmycotrophic, facultatively mycotrophic, and obligately mycotrophic categories, forming a continuum from the least to most responsive mycorrhizal fungi (Allen and Allen 1990). Studies conducted along successional sequences have demonstrated that early-seral plant species are often nonmycorrhizal, followed by facultative and late-seral obligate species (Janos 1980). Interestingly, many pioneer species in the Chenopodiaceae, Brassicaceae, and Amaranthaceae (Gerdeemann 1968) are nonmycotrophic and persist on disturbed soils where mycorrhizal inoculum densities are low (Allen and Allen 1980). For most restoration projects, the goal is to establish late-seral plant species in early-seral soils. The appropriate mycorrhizal association must be considered. Because mycorrhizae are generally adapted to local plant populations (Weinbaum et al. 1996), using locally adapted seeds for the revegetation of a particular species could facilitate mycorrhiza formation. Transplanting mycorrhizal seedlings onto disturbed sites can quickly increase AM infectivity because fungal hyphae expand into open habitats and along roots, slowly spreading the association to adjacent plants (Warner and Mosse 1980).

Manipulating the spatial arrangement of transplants to concentrate resources and create resource islands may provide greater benefits than less intensive treatments over a large area (Allen 1988; Allen and MacMahon 1985). These resource islands can provide seed and inoculum for surrounding areas. Replanting multiple shrubs in a clumped pattern enhances mycorrhizal recovery by trapping more wind-carried propagules between plants than would be deposited around individually spaced plants (Allen et al. 1997).

8 USE OF MYCORRHIZAL INOCULUM

8.1 When is it Appropriate?

Over the past three decades, there has been increasing interest in using mycorrhizal inoculation in large-scale plant production situations, including manipulating and managing the effectiveness of plant–fungus relationships (Miller et al. 1994). Before the selection and culture of fungi begins, it should be determined that inoculation is more appropriate as a management option than manipulation of the native mycorrhizal population. Identifying whether a disturbed site would respond favorably to AM or EM inoculation is the first step. This involves knowing the limitations to plant growth or establishment in a particular soil, and determining whether mycorrhizal fungi can alleviate those restrictions (Dodd and

Thomson 1994). Large-scale disturbances always change soil characteristics, alter plant communities, and reduce mycorrhizal abundance and diversity. Where native fungi have low colonization capacity, but provide benefits to host plants, managing to increase the abundance of fungal populations may be more appropriate than augmenting with nonnative fungi (Dodd and Thomson 1994). Most areas to be restored vary greatly from their predisturbance state. Native ecotypes may or may not be better adapted to the prevailing site conditions (Azcón-Aguilar and Barea 1997). Consequently, using nonnative mycorrhizae better adapted to the current environmental conditions is an important consideration. For instance, management for native populations of mycorrhizal fungi may not be appropriate where exotic trees are planted on disturbed sites. Dunstan et al. (1998) noted that the first attempts to establish pine plantations, especially *Pinus radiata*, in Western Australia were large-scale failures, and it was not until inoculation with compatible EM fungi that pines were successfully introduced. Nevertheless, after 100 years of successive inoculation with exotic EM, the diversity of fungi colonizing roots of pines in plantations remains low, attributed to the host-specific nature of some EM fungi. Another important consideration for inoculating with mycorrhizal fungi is the ability of the symbiosis to reduce the use of fertilizers and pesticides. The use of fungi to reduce fertilizer and pesticide application has been achieved in plant production systems for agriculture, horticulture, and recently, ecosystem restoration (Azcón-Aguilar and Barea 1997). Maximum benefits will only be obtained from careful selection and inoculation of compatible host–fungus–soil combinations (Azcón-Aguilar and Barea 1997).

8.2 Isolating and Culturing Fungi

Despite the benefits of mycorrhizal associations, application has not been widely used on a commercial scale. For commercial development, large quantities of inoculum must be produced. Since AM are obligate biotrophs, they must be grown and maintained on living plant roots. This is often problematic because of the high risk of introducing plant pathogens and other contaminants into the culture system (Jarstfer and Sylvia 1997). In contrast, EM fungi may grow on agar media in the absence of a host plant root or from the vegetative mycelium of fruiting bodies collected directly from the field (Molina and Palmer 1982).

The first step in culturing mycorrhizal fungi is producing stock of the individual fungal isolate on host plant roots. For AM fungi, spores or colonized root fragments from the stock are used to produce larger quantities of inoculum for growth on soil-based or soil-free substrates (Schenck and Perez 1990). Although large amounts of EM fungal spores can be easily collected in the field, spores are rarely used to isolate EM fungi. Instead, many inoculation programs use the EM vegetative mycelium for its effective growth and storage on agar (Molina and Palmer 1982).

There are several benefits to using a soil-based culture system. Soil-based inocula are easy to produce, highly infective, and can be stored for several months or years. Jarstfer and Sylvia (1997) outline the most basic procedure for isolating and culturing spores on plants in sterile soil. Host plants propagated from seed are preferred over cuttings because they are easily disinfected. Disinfecting fungal propagules prior to inoculation is also critical because other microorganisms may be propagated with or instead of the AM fungi (Jarstfer and Sylvia 1997). To avoid contamination, cultures should be isolated from nonsterile environments. Cultures are typically grown for 4–6 months, ensuring sporulation of all genera (Sieverding 1991), and are then stored as air-dried soil at room temperature (Dodd and Thomson 1994). Fungal inoculum can be stored for long periods (>5 years) as air-dried soil; however, the viability of individual isolates during storage remains uncertain (Jarstfer and Sylvia 1997). The most convenient use of soil-based systems has been for inoculating nursery grown plants that are later transplanted in the field. This type of culture system may be otherwise too cumbersome for extensive use on a landscape-scale (Jarstfer and Sylvia 1997).

Soil-free systems, like hydroponics and aeroponics, were developed to overcome the limitations and drawbacks associated with soil-based systems. Culturing fungi in soil-less media provides greater control over the physical and chemical characteristics of the growth medium and minimizes the detrimental impacts of contamination with other organisms (Jarstfer and Sylvia 1995). As such, the ideal conditions conducive to AM development are capable of being achieved. Better control of nutrients in soil-less systems can result in greater root proliferation and higher numbers of spores per centimeter of colonized root length (Sharma et al. 2000). Colonized roots and spores free of substrate allow for more efficient production and distribution of inocula (Jarstfer and Sylvia 1997). Consequently, soil-free systems produce greater propagule densities than soil-based pot cultures of the same age (Jarstfer and Sylvia 1995). Aeroponic culture in particular allows for easy extraction of AM fungal propagules, and mycorrhizal roots can be sheared to produce high-density inoculum that is both efficient and easy to handle (Jarstfer and Sylvia 1995). Commercial nurseries currently use aeroponic culture systems for revegetation programs where on-site production of inocula allows for the use of fresh mycorrhizal propagules at optimal times (Jarstfer and Sylvia 1995).

8.3 Inoculum Diversity

Inoculating with several AM or EM fungal isolates may be appropriate for reestablishing a range of mycotrophic plant species on a disturbed site, especially if plant diversity was high, prior to disturbance (Dodd and Thomson 1994). Whether fungal inocula are native, exotic, or both depends largely on the host plant and environmental conditions. Mixed mycorrhizal inocula may ensure the persistence of inoculant

fungi in the field after transplantation if several isolates were better adapted to conditions in the nursery, rather than extremes in the environment (Dodd and Thomson 1994). Under most circumstances, attempts to increase mycorrhizal fungi in soil have involved inoculation with exotic fungal species (Miller et al. 1994). Exotic fungi must outcompete native mycorrhizal populations, persist on roots, and colonize the root systems of neighboring hosts to be effective. Consequently, most exotic AM and EM fungi are eventually replaced with native mycorrhizae over time (Marx and Cordell 1987). However, their importance to the initial stages of plant establishment is often critical.

8.4 Methods for Applying Inoculum

Most commercially available AM bulk inoculum is a mixture of spores, colonized roots, hyphae, and the substrate on which pot cultures were grown. For EM fungi, many inoculation programs have successfully used the EM vegetative mycelium. The production of mycelial inoculum for large-scale inoculation programs is often costly and pure culture isolates can be difficult to maintain (Marx and Kenney 1982). In contrast, EM sporocarps contain a significant amount of spores that can be collected from the fruiting populations and easily dried and stored until application. Spores from EM fruiting bodies of *Pisolithus*, *Scleroderma*, and *Rhizopogon* are most frequently used as inoculum. Spores of *Laccaria*, *Descolea*, *Scleroderma* and *Pisolithus* spp. have recently been proposed as candidates for nursery inoculation programs for eucalypti (Lu et al. 1998) and *Rhizopogon* spp. are commonly used to inoculate Douglas-fir seedlings in commercial nurseries (Castellano 1994).

The amount, timing, and method of inoculation are important factors in properly managing the establishment of mycorrhizae. Little is known about appropriate application rates. Estimates of 1–2 kg of bulk soil inoculum (5000–10,000 propagules) have been used for AM inoculations (Lovato et al. 1995) and spore suspensions at densities of 10^6 – 10^7 spores per ml have been used for EM inoculations (Lu et al. 1998). The earlier plants are inoculated with fungi, the greater the benefits are to those plants. Inoculation is typically applied in either by broadcasting at the soil surface or by banding inoculum at the root zone. Broadcasting requires more inoculum whereas banding concentrates inoculum near the area of developing roots. Transplanting mycorrhizal seedlings can also be used to inoculate other plant roots. Fungal hyphae expand into open habitats and along roots, slowly spreading the association to adjacent plants (Warner and Mosse 1980).

9 MYCORRHIZAE AND BIOREMEDIATION

In the mining process, not only plants are removed, but also upon soil replacement changes in texture and deposits of salts and heavy metals often result (Allen 1989). The toxicity of

metals not only depends on their concentration in the soil, but also their availability and transfer to plants. By providing a direct link between soil and plants, mycorrhizal function could be of great importance in heavy metal polluted soils (Leyval et al. 1997). As such, there is increasing interest in the potential for mycorrhizal fungi to be used as bioremediation agents or as bioindicators of heavy metal pollution.

It is generally believed that pollution inhibits the formation of mycorrhizal associations. However, little is known about the viability and activity of AM and EM fungi in soils with different heavy metal concentrations. Rühling and Söderström (1990) reported that the number of fruiting bodies and species decreased with increasing pollution along a heavy metal pollution gradient in Sweden. Isolates of *P. tinctorius* collected from old mining sites expressed increased aluminum tolerance and high mycelial growth when compared to isolates from rehabilitated and forested sites (Egerton-Warburton and Griffin 1995). For AM fungi, there is evidence to suggest that at least some AM fungi are relatively resistant to high metal concentration. Gildon and Tinker (1981) reported that plant roots growing naturally on zinc- and cadmium-contaminated soils had significant AM colonization. Davies et al. (2001) reported high rates of AM colonization even at the most toxic levels of chromium in soils. And Rao and Tak (2001) found a significant improvement in root colonization and spore density of AM fungi isolated from gypsum mine spoils when used to inoculate five tree species growing in gypsum mine soils.

The mechanism that confers heavy metal tolerance in mycorrhizal fungi is largely unknown. The survival of AM and EM fungi in polluted soil may depend heavily on the density of the external hyphae. The absorption of heavy metals to the hyphal surface could reduce soil concentrations and thus accumulation of fungal and plant tissue (Denny and Wilkins 1987; Marschner and Dell 1994). Components of the fungal cell wall, such as chitin and melanin, can bind heavy metals to the extraradical mycelium (Denny and Wilkins 1987; Tam 1995). Turnau et al. (1996) found that the EM fungal mantle contained the highest levels of heavy metals while the Hartig net contained the lowest levels. Glomalin, the glycoprotein that coats AM fungal hyphae, could play an equally important role in protecting AM fungi and host plants from toxic metal concentrations in soils, although this has not yet been investigated. Other possible mechanisms conferring heavy metal tolerance in fungi may include intracellular chelation (Martin et al. 1994) and the sequestration of metals within mycorrhizal sheaths (Egerton-Warburton et al. 1993). The stability of metal tolerance in both AM and EM fungi remains to be examined (Leyval et al. 1997). Despite these uncertainties, isolation of tolerant fungal ecotypes that occur on polluted soils could have important application to the revegetation or inoculation of barren polluted sites (Gildon and Tinker 1981; Rao and Tak 2001).

The benefit of heavy metal tolerance in mycorrhizal fungi could have direct effects on host plant response to metal concentrations in soil (Meharg and Cairney 2000). Several studies have reported the beneficial role of EM associations in

reducing metal concentrations in plant tissues. (Colpaert and Van Assche 1992;1993; Dixon and Buschena 1988). Ectomycorrhizal fungi can differ in their ability to reduce translocation from root to shoot, so the presence of the appropriate EM fungi may be critical (Denny and Wilkins 1987). The role of AM fungi in heavy metal uptake is harder to elucidate because of the obligate nature of the fungal symbiont. Regardless of their ability to grow in polluted soils, the extent to which AM fungi confer metal tolerance in their host plants, or accumulate heavy metals in roots preventing translocation to shoots is not fully understood (Leyval et al. 1997). Species of both AM and EM fungi differ in hyphal productivity and in their ability to take up and transfer metals. The turnover of fungal tissue could be an important factor in the ability of mycorrhizal fungi to protect host plants against prolonged elevated metal concentrations (Colpaert and Van Assche 1993).

10 MYCORRHIZAE AND BIOTECHNOLOGY

Despite the promise (Wood and Cummings 1992; Podila and Douds 2000), there has been remarkably little real breakthrough in using mycorrhizae for applied activities, beyond the early work of inoculation for nutrient enhancement. Technologies for inoculation are available (Brundrett et al. 1996), but selection for desirable characteristics and matching plant and fungal genotypes may prove to be crucial. In many cases, if not most, agricultural technologies actually select for populations of fungi that may be detrimental for the crops grown (Johnson et al. 1997). Many of the EM and virtually all of AM fungi are generalists, with the ability to invade a wide range of host plants, but with highly variable response variables (Johnson et al. 1997). Bever et al. (2001) have shown how less-than-optimal taxa can persist in a population and even come to dominate.

Large investments are being made in developing transgenic crops. However, no efforts are underway to even determine if compatibilities or response matches are even important! Kaldorf et al. (2001) have shown that genetically-modified hybrid aspen showed no differences from non-transgenic races in AM or EM infection, and only marginal changes in the fungal community composition were observed. We have also found that transgenic corn had no effect on the species composition or AM infection (Snyder and Allen, unpublished data). Hiremath and Podila (2000) reviewed efforts that demonstrate that genetic transformations of mycorrhizal fungi are possible. Certainly, genes are rapidly being identified and studied (Maldonado-Mendoza et al. 2001) and genes can be added to individual fungi. However, to our knowledge, no transgenic mycorrhizal fungi have been successfully tested and no effects of differing fungi on host responses, either positive or negative, have been evaluated.

This remains an important area of study. Experiences with fungal virus' have shown that extranuclear genetic material can dramatically affect fungal-plant interactions (Roane et al.

1986) and mycorrhizal fungi are no different from others in this trait (Douhan et al. 2003).

11 CONCLUSION

Mycorrhizal fungi are virtually ubiquitous in their evolutionary associations with plants. All trees used for fuel, fiber, and food form mycorrhizae with a diverse array of fungi. All crops, excepting a small number of annuals, and all horticultural plants that decorate our homes and gardens form mycorrhizae. Known benefits range from soil stabilization, to fertilizer and pesticide reduction, to enhanced tolerance of pollutants. These fungi also sequester toxins and could be extremely important in land rehabilitation as well as restoration of lands for grazing or conservation.

Disturbance to plant communities and soil systems can reduce the density, infectivity, and function of mycorrhizae. Without the appropriate fungi present, the natural re-establishment of this beneficial symbiosis may take decades or may never occur. Because of the influential role mycorrhizae play in facilitating plant succession, it is important to understand the factors that regulate mycorrhizal recovery, and to determine how to effectively manage those factors to enhance existing or inoculated fungal populations. For instance, if a disturbance can be anticipated, topsoil preservation could minimize adverse effects to mycorrhizal fungi. If topsoil is lost, or if soil structure is severely altered, soil organic amendments could potentially enhance mycorrhizal development. The ability of mycorrhizal propagules to colonize disturbed sites from across relatively long distances supports the need to maintain source areas with diverse and abundant mycorrhizal populations. In natural disturbances, mycorrhizal propagules disperse by wind and animals. Evaluating host plant characteristics or manipulating the spatial pattern of planting with respect to these two factors can enhance the rate of mycorrhizal recovery (Allen et al. 1997).

Managing a site to facilitate native fungal reinvasion may be less effective if the area varies greatly from its predisturbance state. Native ecotypes may or may not be better adapted to the prevailing site conditions. For instance, in soils polluted by heavy metals, the survival of mycorrhizal fungi could make them important bioindicators. Their ability to function in heavy metal polluted soils may also confer some degree of tolerance in host plants. In contrast, if native ecotypes are particularly sensitive to changes in the soil environment, inoculating with nonnative mycorrhizae better adapted to the current soil conditions is an important consideration. It should, however, first be determined whether the disturbed site would respond favorably to AM or EM inoculation. This involves knowing the limitations of plants and mycorrhizae in particular soils. Actively restoring the mycorrhizal fungal community is an essential component to the success of any restoration program.

The value of mycorrhizal symbioses has been documented for over a century. However, application of mycorrhizae in current biotechnology techniques remain as ignorant as

agronomic and horticultural enterprises that developed practices that reduced or eliminated mycorrhizal benefits in an oversimplified view of the dynamics of soil systems. Mycorrhizal fungi are being studied at the molecular to biochemical level. However, tapping the vast diversity of fungi and alternative mechanisms for increasing yield efficiencies, decreasing pest loss, or rendering polluted landscapes usable has not been studied or extensively utilized. More applied research, especially in developing areas of the world without the financial resources to purchase energy, pesticides, or fertilizers, would pay large dividends.

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